IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	George J. Brewer	Confirmation No.:	1674
Application No.:	10/796,782	Art Unit:	1643
Filed:	March 9, 2004	Examiner:	Maier, Leigh C.
For:	METHODS AND COMPOSITIONS FOR THE TREATMENT OF ANGIOGENIC DISEASES	Docket No:	30275/40847

DECLARATION OF DR. ANDREW P. MAZAR UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, ANDREW P. MAZAR, do declare and state that:

1. I am a citizen of the United States.

2. I presently hold the positions of Chief Scientific Officer and Senior Vice President, Research and Development at Attenuon, LLC. Further positions I have held are set forth in my *curriculum vitae*, attached hereto as Exhibit 1.

3. I received the degree of Bachelor of Science in Chemistry from University of Wisconsin – Parkside in 1987. I received the degree of Doctor of Philosophy in Biochemistry from the University of Illinois College of Medicine in 1993.

4. My education, technical experience and professional activities, honors and awards, and list of recent publications are set forth in my *curriculum vitae*, attached hereto as Exhibit 1. I have worked extensively in the areas of tumor progression and angiogenesis.

5. Attenuon, LLC is a licensee of the above-identified patent application.

6. I understand that the claims as amended are directed to a method of treating a disease characterized by ocular neovascularization in an animal, comprising orally administering to an animal having a disease characterized by ocular neovascularization a loading dose of greater than 200 mg daily of a thiomolybdate compound that binds copper and forms an

agent-copper-protein complex. I understand that claims of the above-identified patent application ("the '782 application") are subject to a rejection by the Examiner based at least in part upon the Examiner's contention that treatment of the recited diseases is not described in the specification in a way to teach one of ordinary skill in the art how to make and use what is claimed.

7. The following experiments were carried out by me or under my supervision. These experiments indicated that oral administration of tetrathiomolybdate (TM) inhibits choroidal neovascularization (CNV) in two models of advanced macular degeneration (AMD).

8. First, the effects of TM were evaluated in an early treatment laser-induced model of AMD. Female C57BL/6J mice were anesthesized and their pupils dilated. Three burns of 532 nm diode laser photocoagulation were delivered to each retina. Burns were performed in the 9, 12, and 3 o'clock positions of the posterior pole of the retina. Mice were treated orally every morning with 10 mg/kg of TM (15 mice) or phosphate buffered saline (PBS) (10 mice) from day 1 to day 13. On day 14, the mice were euthanized and analyzed.

9. Exhibit 2 shows the result of the experiment described in paragraph 8. Approximately 50% inhibition of angiogenesis, observed as an inhibition of CNV area, was observed with the TM treatment.

10. Second, periocular injections of TM were administered instead of the oral administrations in the same early treatment model described in paragraph 8.

11. The results of the experiment described in paragraph 10 showed no difference in the prevention of choroidal neovascularization between TM and the vehicle control by periocular administration (data not shown).

12. Third, the effect of TM was evaluated in a therapeutic laser-induced model of AMD. The protocol was as described above in paragraph 8, except that treatment was not initiated until day 14 after laser injury, when CNV was already established.

13. Exhibit 3 shows the result of the experiment described in paragraph 12. A trend towards regression of CNV was observed. I believe that an enhanced regression of CNV would be observed if higher dosages of TM had been used, in view of the trend observed in Exhibit 3 and the data achieved with a higher dose in the experiment described in paragraphs 16 and 17 below.

14. Thus, the results in Exhibit 2 demonstrate inhibition of CNV using TM. The results in Exhibit 3 indicate that orally administering tetrathiomolybdate to an animal results in regression of choroidal neovascularization.

15. The following additional experiment was carried out by me or under my supervision. These experiments demonstrated that ATN-224, the bis-choline salt of tetrathiomolybdate, inhibits angiogenesis in a MATRIGEL™ plug model in mice. The MATRIGEL™ plug model is a general model of angiogenesis without regard to a specific disease state and is often used to identify and screen antiangiogenic agents.

16. This Matrigel plug model was performed essentially as described by Passaniti *et al.*, 1992, Lab Invest. 67:519-528 (Exhibit 4). In this model, FGF-2 induces angiogenesis. Ice-cold MATRIGEL™ (Collaborative Biomedical Products, Inc., Bedford, MA) was mixed with heparin (50 μg/ml) and FGF-2 (300 ng/ml). The MATRIGEL™ mixture (500 μL/mouse) was injected subcutaneously into 4-8 week-old athymic nude mice at sites near the abdominal midline, preferably 2 injections per mouse. The injected MATRIGEL™ forms a palpable solid gel. Injection sites were chosen such that each animal receives a FGF-2 plug (300 ng/ml) and a negative control plug (MATRIGEL™ only). ATN-224 (50 mg/kg) or a vehicle control (water) was administered orally by gavage qd to the mice beginning on day 5 after injection of the MATRIGEL™ plug. Animals were sacrificed by cervical dislocation on day 4 (to evaluate the extent of angiogenesis prior to initiating ATN-224 treatment) or day 9 post injection. The mouse skin was detached along the abdominal midline, and the MATRIGEL™ plugs were recovered and scanned immediately at high resolution. Plugs were then dispersed in water and incubated at 37°C overnight. Hemoglobin (Hb) levels were determined using Drabkin's solution (*e.g.*, obtained from Sigma) according to the manufacturers' instructions. The amount of Hb in the plug has been validated as indirect measure of angiogenesis in this model. See Passaniti *et al.*, 1992, Lab Invest. 67:519-528.

17. Exhibit 5 shows the results of the experiment described in paragraph 16. ATN-224 dramatically regressed established neovessels stimulated by FGF-2. Similar results were also observed when angiogenesis was stimulated by VEGF instead of FGF-2 in this model (data not shown). This is in contrast to many other antiangiogenic agents that are only able to prevent neovessel formation but are unable to regress established neovessels. See, *e.g.*, Nambu *et al.*, 2003, Invest. Ophthalmol. Vis. Sci. 44:3650-3655 (Exhibit 6) at page 3652, paragraph spanning cols. 1 and 2.

18. Both ATN-224 and TM are thiomolybdate compounds that form thiomolybdate compound-copper-protein complexes. I reasonably expect that thiomolybdate compounds that form thiomolybdate compound-copper-protein complexes, in view of their structural similarities, generally would display effects upon angiogenesis and CNV as indicated by the results in Exhibits 2, 3 and 5.

19. In summary, the experiment described in paragraphs 16-17 demonstrates that a thiomolybdate compound inhibits angiogenesis and regresses established neovessels. The experiment described in paragraphs 8-9 demonstrates that a thiomolybdate compound inhibits CNV. The results in Exhibit 5 suggest that a thiomolybdate compound may cause regression of CNV.

20. Diseases characterized by ocular neovascularization, including those associated with choroidal, corneal and/or retinal neovascularization, all involve aberrant angiogenesis. Neovascularization in the eye can result in new blood vessels that can grow into nearly all mature ocular tissue, including the cornea and retina, resulting in many eye diseases. See Lee *et al.*, 1998, Surv. Ophthamol. 43:245-269 (Exhibit 7) at page 245, col. 2. This common mechanism provides a rationale for treating all diseases characterized by ocular angiogenesis with anti-angiogenic agents, such as thiomolybdate compounds. Further, in a study directed at answering the question "Do agents that inhibit retinal neovascularization have any effect on choroidal neovascularization?", an agent shown to inhibit retinal neovascularization also resulted in dramatic inhibition of choroidal neovascularization. See Seo *et al.*, 1999, Am. J. Pathol. 154:1743-1753 (Exhibit 8), abstract. The authors conclude that "pharmacological treatment is a viable approach for therapy of both retinal and choroidal neovascularization." Based on the common mechanisms behind ocular neovascularization and experimental data, one of skill in the art would reasonably expect that an anti-angiogenesis agent useful in one type of ocular neovascularization could be used for all types of ocular neovascularization. This prediction has been successfully demonstrated in various additional experiments. For example, anti-VEGF agents have been used in the clinic for the inhibition of more than one of choroidal neovascularization, corneal neovascularization and retinal neovascularization. See Wegewitz *et al.*, 2005, Current Pharmaceutical Design 11:2311-2330 (Exhibit 9) at page 2321, col. 1, paragraphs 2 and 3 and page 2322, col. 2, paragraph 4; van Wijngaarden *et al.*, 2005, JAMA 293:1509-1513 (Exhibit 10) at page 1510, col. 1 to page 1511, col. 1. Inhibitors of C-Raf kinase have shown decreased choroidal and retinal neovascularization in

animal models. See Henry *et al.*, 2004, Trends in Pharm. Sci. 25:523-527 (Exhibit 11) at page 526, col. 1, first paragraph.

21. Thus, one of skill in the art would expect that an agent that inhibits one type of ocular neovascularization would be useful for inhibiting other types of ocular neovascularization.

22. In view of the foregoing, I conclude, and believe that others skilled in the art would also conclude, that a thiomolybdate compound can be used generally to treat diseases characterized by ocular neovascularization when orally administered at a loading dose of greater than 200 mg daily.

23. I declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 6/19/06

ANDREW P. MAZAR

Attachments:

Exhibit 1: *Curriculum Vitae* of Andrew P. Mazar

Exhibit 2: Inhibition of choroidal angiogenesis in an early treatment laser-induced model of AMD

Exhibit 3: Inhibition of choroidal angiogenesis in a therapeutic laser-induced model of AMD

Exhibit 4: Passaniti *et al.*, 1992, Lab Invest. 67:519-528

Exhibit 5: ATN-224 regresses neo-vessels in a Matrigel Plug model of angiogenesis

Exhibit 6: Nambu *et al.*, 2003, Invest. Ophthalmol. Vis. Sci. 44:3650-3655

Exhibit 7: Lee *et al.*, 1998, Surv. Ophthamol. 43:245-269

Exhibit 8: Seo *et al.*, 1999, Am. J. Pathol. 154:1743-1753

Exhibit 9: Wegewitz *et al.*, 2005, Current Pharmaceutical Design 11:2311-2330

Exhibit 10: Wijngaarden *et al.*, 2005, JAMA 293:1509-1513

Exhibit 11: Henry *et al.*, 2004, Trends in Pharm. Sci. 25:523-527

CURRICULUM VITAE

Andrew Paul Mazar
1608 India St. #403
San Diego, CA 92101
(619) 546-6619

June 2006

DEGREES AWARDED

Ph.D., Biochemistry
University of Illinois College of Medicine
Department of Biochemistry, Chicago, Illinois

B.S., Chemistry (honors), concentration in Biochemistry
University of Wisconsin – Parkside, Kenosha, Wisconsin

EMPLOYMENT EXPERIENCE

February 2000 to Present	*Chief Scientific Officer and Senior Vice President, Research and Development* Attenuon, LLC, San Diego, California
February 2006-	*Adjunct Professor of Research* University of Texas Health Center at Tyler, Tyler, Texas
April 1999 to February 2000	*Vice President, Biology* Ångstrom Pharmaceuticals, Inc., San Diego, California
January 1999 to March 2003	*Adjunct Professor of Medicine* University of North Texas Health Science Center, Fort Worth, Texas
November 1998 to April 1999	*Senior Director, Biology* Ångstrom Pharmaceuticals, Inc., San Diego, California
May 1997 to November 1998	*Director, Tumor Biology* Ångstrom Pharmaceuticals, Inc., San Diego, California
January 1997 to May 1997	*Consultant* Ångstrom Pharmaceuticals, Inc., San Diego, California
September 1996 to May 1997	*Lecturer* Department of Biology, Barat College, Lake Forest, Illinois
September 1993 to	*Adjunct Assistant Professor of Medicine*

EMPLOYMENT EXPERIENCE

January 1998	Northwestern University College of Medicine, Chicago, Illinois
January 1996 to November 1996	*Group Leader, Anti-metastasis/Anti-angiogenesis Research* Abbott Laboratories, Abbott Park, Illinois
April 1992 to January 1996	*Senior Research Biochemist, Thrombolytics Venture/Anti-metastasis Research* Abbott Laboratories, Abbott Park, Illinois
April 1989 to April 1992	*Research Biochemist, Thrombolytics Venture/Anti-metastasis Research* Abbott Laboratories, Abbott Park, Illinois
1987 to 1990	*Teaching Assistant and Tutor* Department of Medicine, University of Illinois College of Medicine, Chicago, Illinois
1984 to 1987	*Hospital Corpsman* United States Navy, Great Lakes, Illinois

HONORS AND AWARDS

2005	Guest Editor, Current Cancer Drug Targets, Hot Topics Issue on Novel Targets for Anti-angiogenic and Cytostatic therapies
2005	Organizing Committee – International Congress on Fibrinolysis and Proteolysis (2006)
2003	Chairperson, AACR Annual Meeting, Symposium: *Expression of Metastasis-related Genes*
	Co-Chairperson, AACR Annual Meeting, Subsection BL3: *Tumor Progression, Invasion and Metastasis*
2001	Invited Participant, Banbury Center/Cold Spring Harbor: *New Concepts for Cancer Clinical Trials*
	Invited participant, ASCO Annual Meeting, Angiogenesis Expert Panel
1999 to 2003	Advisory Board Member, Institute for Cancer Research, University of North Texas Health Science Center, Fort Worth, Texas
1995	Candidate Interviewer, Northwestern Medical School, Chicago, Illinois

HONORS AND AWARDS

1993	Scientific Advisor, Waukegan School District 60, Waukegan, Illinois
1991	Young Investigator Award, 1st Osteosarcoma Research Conference, Allegheny-Singer Research Institute, Pittsburgh, Pennsylvania
1989	NSF Travel Grant
	Selected NATO Fellow to attend NATO sponsored Advanced Study Institute: *The enzyme catalysis process: energetics, mechanism and dynamics*, Barga, Italy
1988	University of Illinois Graduate College Fellowship, University of Illinois College of Medicine, Department of Biochemistry, Chicago, Illinois
1987	Honors Awarded in Chemistry, University of Wisconsin – Parkside, Kenosha, Wisconsin

PROFESSIONAL SOCIETIES

American Society for Biochemistry and Molecular Biology

American Association for Cancer Research

American Association for the Advancement of Science

American Society of Clinical Oncology

Protein Society

AD HOC EDITORIAL REVIEW

Blood

BMC Cancer

Breast Cancer Research

British Journal of Cancer

Cancer Research

Ad Hoc Editorial Review

Clinical Cancer Research

Experimental Cell Research

FEBS Journal

Gene Therapy

Expert Opinion on Therapeutic Patents

International Journal of Cancer

Journal of Histochemistry and Cytochemistry

Medicinal Research Reviews

Molecular and Cellular Biochemistry

Molecular Cancer Research

Oncology Research.

Editorial Boards

Recent Patent Reviews on Anti - Cancer Drug Discovery

COMMITTEES AND STUDY SECTIONS

2006	NIH/NIDDK ZDK1 GRB4 O1&O2: STTR/SBIR
2005	NIH/NIDDK Special Emphasis Panel/SRG 2005/10 ZDK1 GRB-4 (O1)
2004	Phillip Morris External Research Program
2003	NIH/NHLBI SCCOR Study Panel ZHL1 CSR-R M1 R
2002	NIH/SBIR Study Section ZRG1 SSS-2
2001	NIH/SBIR Study Section ZRG1 SSS-2
	VA Oncology Merit Review Study Section
2000	VA Oncology Merit Review Study Section
1999	Co-Chairperson, NCI/NHLBI Workshop on Fibrin Turnover in Inflammation and Neoplasia
	VA Oncology Merit Review Study Section
	American Heart Association, Ad Hoc Grant Reviewer

BOOK CHAPTERS

Mazar, A.P., Henkin, J., Goltzman, D. and Rabbani, S.A. (1991) "A N-terminal fragment of urinary plasminogen activator which is mitogenic in SaOS-2 cells is generated proteolytically by PC-3 cells." in Frontiers of Osteosarcoma Research, J.F. Novak and J.H. McMaster, eds. Toronto: Hogrefe and Huber, pp. 319-322.

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Rabbani, S.A., Shukeir, N., Mazar, A.P (2004). "Prostate Cancer: Models for Developing Novel Therapeutic Approaches." in Bone Metastasis and Molecular Mechanisms: G. Singh and F.W. Orr (Eds.), Kluwer Academic Publishers (Boston): 163-186.

Parry, G.C., Doñate, F., Plunkett, M.L., Shaw, D.E., Pirie-Shepherd, S. and Mazar, A.P. (2005) "Integrins: Novel targets for the treatment of bone metastasis." in Bone Metastasis: Experimental and Clinical Aspects. S. Rabbani and G. Singh (Eds.) Humana Press: 201-228.

PUBLICATIONS

Rabbani, S.A., Desjardins, J., Bell, A.W., Banville, D., Mazar, A., Henkin, J. and Goltzman, D. (1990) "An amino-terminal fragment of urokinase isolated from a prostate cancer cell line (PC-3) is mitogenic for osteoblast-like cells." Biochem. Biophys. Res. Comm. 173(3): 1058-1064.

Hoosein, N.M., Boyd, D.D., Hollas, W.J., Mazar, A., Henkin, J. and Chung, L.W.K. (1991) "Involvement of urokinase and its receptor in the invasiveness of human prostatic carcinoma cell lines." Cancer Comm. 3(8): 255-264.

Mazar, A.P., Buko, A., Petros, A.M., Barnathan, E.S. and Henkin, J. (1992) "Domain analysis of urokinase plasminogen activator (uPA): Preparation and characterization of intact A-chain molecules." Fibrinolysis 6 (suppl.1): 49-55.

Hollas, W., Soravia, E., Mazar, A., Henkin, J., Blasi, F. and Boyd, D. (1992) "Decreased urokinase receptor expression by overexpression of the plasminogen activator in a colon cancer cell line." Biochem. J. 285: 629-634.

Zini, J.M., Murray, S.C., Graham, C.H., Lala, P.K., Barnathan, E.S., Mazar, A., Henkin, J., Cines, D.B. and McCrae, K.R. (1992) "Characterization of urokinase receptor expression by human placental trophoblasts." Blood 79(11): 2917-2929.

Hansen, A.P., Petros, A.M., Mazar, A.P., Pederson, T.M., Rueter, A. and Fesik, S.W. (1992) "A practical method for uniform isotopic labeling of recombinant proteins in mammalian cells." Biochemistry 31(51): 12713-12718.

Rabbani, S.A., Mazar, A.P., Bernier, S.M., Haq, M., Bolivar, I., Henkin, J., and Goltzman, D. (1992) "Structural requirements for the growth factor activity of the amino-terminal domain of urokinase." J. Biol. Chem. 267(20): 14151-14156.

Novokhatny, V., Medved, L., Mazar, A., Marcotte, P., Henkin, P. and Ingham, K. (1992) "Domain structure and interactions of recombinant urokinase-type plasminogen activator." J. Biol. Chem. 267(6): 3878-3885.

Hollas, W., Hoosein, N., Chung, L.W.K., Mazar, A., Henkin, J., Kariko, K., Barnathan, E.S. and Boyd, D. (1992) "Expression of urokinase and its receptor in invasive and non-invasive prostate cancer cell lines." Thromb. and Haemos. 68(6): 662-666.

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Clayman, G., Wang, S.W., Nicolson, G.L., El-Naggar, A., Mazar, A., Henkin, J., Blasi, F., Goepfert, H. and Boyd, D.D. (1993) "Regulation of urokinase-type plasminogen activator expression in squamous cell carcinoma of the oral cavity." Int. J. Cancer 54: 73-80.

PUBLICATIONS (CONT.)

Hollas, W., Kariko, K., Barnathan, E., Mazar, A., Henkin, J. and Boyd, D. (1993) "Divergent effect of N, N-dimethylformamide on the expression of urokinase and its receptor." Fibrinolysis 7: 149-157.

Zhang, S., Laurent, M., Lopez-Alemany, R., Mazar, A., Henkin, J., Ronne, E., and Burtin, P. (1993) "Comparative localization of receptors for plasmin and for urokinase on MCF7 cells." Exp. Cell Res. 207: 290-299.

Novokhatny, V.V., Medved, L.V., Mazar, A. and Ingham, K.C. (1993) "Effect of tethered peptidylchloromethyl-ketone inhibitors on thermal stability and domain interactions of urokinase and other serine proteases." J. Biol. Chem. 268(23): 17211-17218.

Hansen, A.P., Petros, A.M., Meadows, R.P., Nettsesheim, D.G., Mazar, A.P., Olejniczak, E.T., Xu, R.X., Pederson, T.M., Henkin, J. and Fesik, S.W. (1994) "Solution structure of the amino terminal fragment of urokinase-type plasminogen activator." Biochemistry 33: 4847-4864.

Howell, A.L., Hunt, J.A., James, T.W., Mazar, A., Henkin, J. and Zacharski, L.R. (1994) "Urokinase inhibits HL-60 cell proliferation in vitro." Blood Coag. and Fibrinol. 5: 445-453.

Multhaupt, H.A.B., Mazar, A., Cines, D.B., Warhol, M., McCrae, K.R., (1994) "Expression of urokinase receptors by human trophoblast: a histochemical and ultrastructural analysis." Laboratory Investigation 71(3): 392-400.

Schnaper, H.W., Barnathan, E.S., Mazar, A.P., Maheshwari, S., Ellis, S., and Kleinman, H.K. (1995) "Plasminogen activators augment endothelial cell organization in vitro by two distinct pathways." J. Cell. Phys. 165: 107-118.

Rabbani, S.A., Harakidas, P., Davidson, D., Henkin, J. and Mazar, A.P. (1995) "Prevention of prostate cancer metastasis by a urokinase-type plasminogen activator (uPA) specific inhibitor in vivo." Int. J. Cancer 63: 840-845.

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Higazi, A. A-R., Mazar, A., Wang, J., Quan, N., Griffin, R., Reilly, R., Henkin, J. and Cines, D.B. (1997) "Soluble human urokinase receptor is composed of two active subunits." J. Biol. Chem.272 (8): 5348-5353.

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Xing, R.H., Mazar, A., Henkin, J. and Rabbani, S.A. (1997) "Prevention of breast cancer growth, invasion and metastasis by anti-estrogen tamoxifen alone or in combination with urokinase inhibitor B-428." Cancer Res.57: 3585-3593.

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Gum, R., Juarez, J., Allgayer, H., Mazar, A., Wang, Y. and Boyd, D. (1998) "Stimulation of urokinase-type plasminogen activator receptor expression by PMA requires a JNK1-dependent signaling molecule." Oncogene 17: 213-225.

Allgayer, H., Wang, H., Gallick, G. E., Crabtree, A, Mazar, A., Jones, T., Kraker, A. J. and Boyd, D. D. (1999) "Transcriptional induction of the urokinase receptor (uPAR) gene by a constitutively active Src: requirement of an upstream motif (-152/-135) bound with Sp1." J. Biol. Chem. 274: 18428-37.

Mazar, A., Henkin, J. and Goldfarb, R. H. (1999) "The urokinase plasminogen activator (uPA/uPAR/PAI-1) system in cancer: implications for angiogenesis, invasion and tumor metastasis." Angiogenesis 3(1): 15-32.

Guo, Y. J., Arakelian, A., Goldfarb, R. H., Higazi, A. A-R., Jones, T.R., Kwaan, H., Mazar, A.P. and Rabbani, S.A. (2000) "A peptide derived from the non-receptor binding region of urokinase plasminogen activator (uPA) inhibits tumor growth and metastasis and induces tumor cell death in vivo." FASEB J. 14: 1400-1410.

Haj-Yehia, A., Nassar, T., Sachais, B.S., Kuo, A., Bdeir, K., Al-Mehdi, A. B., Mazar, A., Cines D.B. and Higazi, A.A-R. (2000) "Urokinase-derived peptides regulate vascular smooth muscle contraction in vitro and in vivo." FASEB J. 14: 1411-1422.

Shliom, O., Huang, M., Sachais, B., Weisel, J. W., Nagaswami, C., Nassar, T., Bdeir, K., Hiss, E., Gawlak, S., Harris, S., Mazar, A. and Higazi, A.A. (2000) "Novel Interactions Between Urokinase and it Receptor." J. Biol. Chem. 275(32): 24304-12.

PUBLICATIONS (CONT.)

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Mazar, A.P., Gawlak, S., Goldfarb, R.H., Kitson, R.P., Rabbani, S.A., Guo, J., Gown, A. and Jones, T.R. (2000) "The urokinase plasminogen activator receptor (uPAR) is a choke-point for carcinoma metastasis and angiogenesis." Recent Research Developments in Cancer 2: 1-12.

Bdeir, K., Kuo, A., Mazar, A., Sachais, B.S., Xiao, W., Gawlak, S., Harris, S., Higazi, A.A. and Cines, D.B. (2000) "A region in domain II of the urokinase receptor required for urokinase binding." J. Biol. Chem. 275: 28532-28538.

Tarui, T., Mazar, A.P., Cines, D.B., Takada, T. (2001) "Urokinase Receptor (uPAR/CD87) is a genuine ligand for integrins and mediates cell-cell interaction." J. Biol. Chem. 276: 3983-3990.

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Neoplasia." Am. J. Respir. Crit. Care Med. 2001 Feb 1: 163(2): 578-584.

Mazar, A.P. (2001) "The Urokinase Receptor as a Target for the Diagnosis and Therapy of Cancer." Anticancer Drugs 12(5): 387-400.

Al-Atrash, G., Kitson, R., Xue, Y., Mazar, A., Kim, M. and Goldfarb, R. (2001) "uPA and uPAR contribute to NK cell invasion through the extracellular matrix." Anticancer Research 21: 1-8.

Zhang, J.C., Qi, X., Juarez, J., Plunkett, M., Donate, F., Sakthivel, R., Mazar, A.P., McCrae, K.R. (2002) "Inhibition of angiogenesis by two-chain high molecular weight kininogen (HKa) and kininogen-derived polypeptides." Can. J. Physiol. Pharmacol. 80: 85-90.

Guo, Y., Mazar, A.P., Lebrun, J.J. and Rabbani, S.A. (2002) "An Anti-Angiogenic Urokinase Derived Peptide (Å6) Combined with Tamoxifen (TAM) Decreases Tumor Growth and Metastasis in a Syngeneic Model of Breast Cancer." Cancer Res. 15: 4678-4684.

Zhang, J.C., Donate, F., Ziats, N.P., Juarez, J.C., Mazar, A.P., Pang, Y-P., McCrae, K.R. (2002) "The antiangiogenic activity of cleaved high molecular weight kininogen is mediated through binding to endothelial cell tropomyosin." Proc Nat Acad. Sci. 99: 12224-12229.

Juarez, J.C., Guan, X., Shipulina, N., Plunkett, M., Parry, G.C., Shaw, D.E., Zhang, J-C., McCrae, K.R., Mazar, A.P., Morgan, W.T., Donate, F. (2002) "The histidine-proline rich glycoprotein (HPRG) has potent anti-angiogenic and anti-tumor activities mediated through the histidine-proline rich domain." Cancer Res. 62: 5344-5358.

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Donate, F. and Mazar, A.P. "Identification of Novel Protein Targets on the Surface of Stressed Cells" USSN 60/728,757.

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EXHIBIT 2

Inhibition of choroidal angiogenesis in a laser-induced model of AMD. *Female C57Bl/6J mice were anesthetized with ketamine hydrochloride (100 mg/kg body weight) and the pupils were dilated with 1% tropicamide. Three burns of 532 nm diode laser photocoagulation were delivered to each retina. Burns were performed in the 9, 12, and 3 o'clock positions of the posterior pole of the retina. Production of a bubble at the time of laser, which indicates rupture of Bruch's membrane, is an important factor in obtaining CNV, so only burns in which a bubble was produced were included in the study. Mice were treated orally every morning with 20 mg/kg of ATN-427 (15 mice) or 10mg/kg of TM (15 mice) or PBS (10 mice) from day 1 to 13. At day 14, the mice were euthanized and analyzed (* p=0.004; ** p=0.003; statistical tests were performed by t-test).*

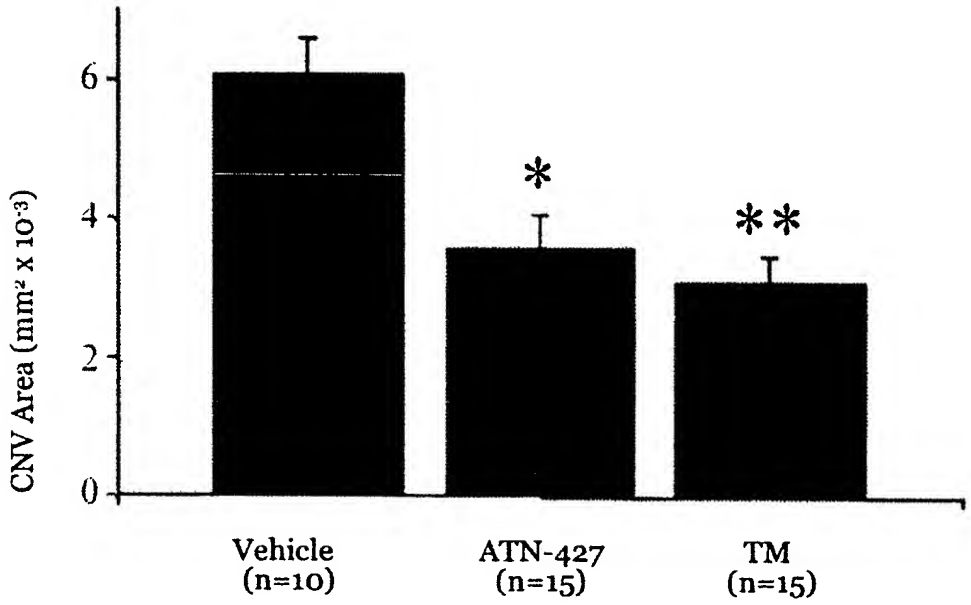
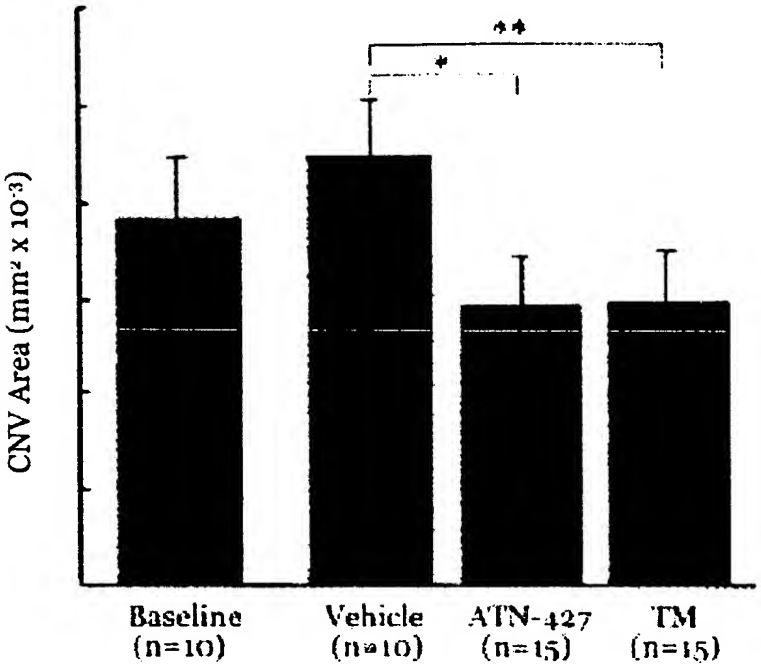


EXHIBIT 3

Inhibition of choroidal angiogenesis in a laser-induced model of AMD. *Protocol was identical to that described in the legend to Exhibit 2 except that treatment was not initiated until day 14 after laser injury followed by 14 days of treatment (* p=0.03; ** p=0.003; statistical comparisons performed by t-test).*



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Methods in Laboratory Investigation

A Simple, Quantitative Method for Assessing Angiogenesis and Antiangiogenic Agents Using Reconstituted Basement Membrane, Heparin, and Fibroblast Growth Factor

ANTONINO PASSANITI, ROBERT M. TAYLOR, ROBERTO PILI, YUE GUO, PETER V. LONG, JOSEPH A. HANEY, REBECCA R. PAULY, DERRICK S. GRANT, AND GEORGE R. MARTIN

Laboratories of Biological Chemistry and Cardiovascular Science, Gerontology Research Center, National Institute on Aging, National Institutes of Health and Department of Pathology and Dermatology, Johns Hopkins Medical Institutions, Baltimore and Laboratory of Development Biology, National Institute of Dental Research, Bethesda, Maryland

BACKGROUND: Blood vessel growth is necessary for normal tissue homeostasis and contributes to solid tumor growth. Methods to quantitate neovascularization should be useful in testing biological factors and drugs that regulate angiogenesis or to induce a vascular supply to promote wound healing.

EXPERIMENTAL DESIGN: An extract of basement membrane proteins (Matrigel) was found to reconstitute into a gel when injected subcutaneously into C57/BL mice and to support an intense vascular response when supplemented with angiogenic factors.

RESULTS: New vessels and von Willebrand factor antigen staining were apparent in the gel 2–3 days after injection, reaching a maximum after 3–5 days. Hemoglobin content of the gels was found to parallel the increase in vessels in the gel allowing ready quantitation. Angiogenesis was obtained with both acidic and basic fibroblast growth factors and was enhanced by heparin. Several substances were tested for angiostatic activity in this assay by coinjection in Matrigel with fibroblast growth factor and heparin. Platelet-derived growth factor BB, interleukin 1-β, interleukin-6, and transforming growth factor-β were potent inhibitors of neovascularization induced by fibroblast growth factor. Tumor necrosis factor-α did not alter the response but was alone a potent inducer of neovascularization when coinjected with Matrigel and heparin. Consistent with the previously demonstrated importance of collagenase in mediating endothelial cell invasion, a tissue inhibitor of metalloproteinases that also inhibits collagenases was found to be a potent inhibitor of fibroblast growth factor-induced angiogenesis.

CONCLUSIONS: Our assay allows the ready quantitative assessment of angiogenic and antiangiogenic factors and should be useful in the isolation of endothelial cells from the capillaries that penetrate into the gel.

Additional key words: Neovascularization, Matrigel

The development of a vascular supply is essential for the growth, maturation, and maintenance of normal tissues (1). It is also required for wound healing (2) and the rapid growth of solid tumors (3, 4) and is involved in various other pathological conditions (1, 5–7). Current concepts of angiogenesis, based in large part on studies on the vascularization of tumors (1), suggest that cells secrete angiogenic factors that induce endothelial cell migration, proliferation, and capillary formation. Although the factors that induce angiogenesis *in situ* are not well identified, numerous factors have been identified that induce vessel formation *in vitro* or *in vivo* in animal models. These include acidic fibroblast growth factor (aFGF) (1, 8, 9), basic (b) FGF (1, 9, 10), transforming growth factor (TGF)-α (1), TNF-α (11, 12), vascular permeability factor or vascular endothelium growth factor (13–15), monobutyrin (16), angiotropin (17), angiogenin (18), hyaluronic acid degradation products (19), and age-associated glycosylation end-products (20). Also, many compounds have been described as inhibitors of angiogenesis including a cartilage-derived inhibitor, identified as tissue inhibitor of metalloproteinases (TIMP) (21, 22), platelet factor-4 (23), thrombospondin (24–26), laminin peptides (27), heparin/cortisone (28–30), minocycline (31), fumagillin (32), difluoromethyl ornithine (33), and sulfated chitin derivatives (34).

The classic assessment of an angiogenic factor is achieved either by embedding the factor in a controlled

release polymer such as ethylene vinyl acetate or cellulose discs (reviewed in Ref. 22) and implanting the substances in the cornea of an animal's eye (35, 36) or by placing these substances on the chorioallantoic membrane of the chick embryo (37) and observing the sprouting of new vessels toward the pellet. Alginate-encapsulated tumor cells (38, 39) and gelatin-impregnated sponges (40) (Gelfoam, Upjohn, Kalamazoo, Michigan) have also been used as angiogenesis inducers. In the alginate tumor cell model, hemoglobin content was used to quantitate angiogenesis. In addition, several *in vitro* models have been used to examine the progression of angiogenesis including the sprouting, attachment, migration, invasion, and morphological differentiation of endothelial cells (8, 12, 41–44).

Here we report on a simple, rapid, and quantitative assay to assess inducers as well as inhibitors of angiogenesis. In brief, we inject a solution a basement membrane proteins supplemented with FGF and heparin subcutaneously in a mouse where it forms a gel. Sprouts from vessels in the adjacent tissue penetrate into the gel within days, connecting it with the external vasculature. Angiogenesis was quantitated by image analysis of vessels and by measuring the hemoglobin present in the vessels within the gel. This assay will facilitate the testing of both angiogenic and angiostatic agents *in vivo* and may allow isolation of the endothelial cells responding to the angiogenic factors for further studies *in vitro*.

EXPERIMENTAL DESIGN

PREPARATION OF ANGIOGENIC FACTORS AND VEHICLE

Liquid Matrigel maintained at 4°C was used as a vehicle to inject angiogenic factors subcutaneously into C57/BL mice. Various components were mixed with liquid Matrigel at 4°C which, when injected into a mouse, formed a single, readily recovered gel. Such gels were removed at various times and processed for histology, total protein, and hemoglobin content.

Matrigel, an extract of murine basement membrane proteins consisting predominantly of laminin, collagen IV, heparan sulfate, proteoglycan, and nidogen/entactin was prepared as a sterile solution as previously described (45). Heparin was dissolved in sterile phosphate-buffered saline (PBS) to 16,000 units/ml. Further dilutions were made with sterile filtered PBS containing 1 mg bovine serum albumin/ml. aFGF (HBGF-1) (R & D, Minneapolis, Minnesota) was diluted to 0.25 μg/ml with PBS/bovine serum albumin. Various amounts of heparin and/or FGF were mixed with 0.5–1.0 ml of Matrigel at 4°C in proportions not exceeding 1% of the volume of Matrigel to be injected. In some cases, other factors were included as noted.

INJECTION AND PROCESSING OF GELS

C57BL mice (five per data point) were each injected subcutaneously with 0.5 ml Matrigel and 0–100 ng aFGF/ml and 0–64 units heparin/ml near the abdominal midline using a 25-gauge needle. The injected Matrigel rapidly formed a single, solid gel that persisted for at least 10 days in the mice. Mice were subsequently killed, and gels were recovered and processed for further studies. Typically, the overlying skin was removed, and gels were cut out by retaining the peritoneal lining for support. For most histological sections, the skin and underlying peritoneum were Formalin-fixed immediately after dissection.

QUANTITATION OF NEOVESSELS

Hemoglobin was measured using the Drabkin method (46) and Drabkin reagent kit 525 (Sigma, St Louis, Missouri). Samples for each point were from five different mice. The concentration of hemoglobin was calculated from a known amount of hemoglobin assayed in parallel. Protein content of the supernatant fluid was determined using the BioRad protein assay method (47). The Optomax image analysis system (Optomax, Hollis, New Hampshire) was used for quantitation of histological specimens by light microscopy (see "Methods").

RESULTS AND DISCUSSION

MATRIGEL AS A VEHICLE FOR ANGIOGENIC FACTORS

In developing a more reproducible and quantitative angiogenic model, we utilized FGFs that are proven and potent inducers of neovascularization. When injected alone subcutaneously into mice, neither aFGF nor bFGF induced any visible signs of neovessel formation (data not shown). This is not unexpected since the factors would be expected to be rapidly cleared from the site. We tested Matrigel, a solution of basement membrane proteins isolated from the Engelbreth-Holm-Swarm tumor, as a vehicle for the slow release of angiogenic factors since it is a liquid at 4°C but forms a gel *in vivo*. Indeed, our studies showed that the gels which formed after subcutaneous injection of Matrigel alone were readily distinguished from surrounding tissue, persisted for at least 10 days, and produced little or no local reaction or angiogenic response (Fig. 1*A*).

Matrigel supplemented with FGF alone produced gels that showed a variable angiogenic reaction (data not shown). Magnitude of the angiogenic response was considerably greater in gels supplemented with both FGF and heparin (Fig. 1*B* and *C*). Subcutaneous injection of Matrigel plus aFGF and heparin at the ventral midline achieved optimal and reproducible responses, whereas material injected either anteriorly or posteriorly to the midline resulted in less consistent responses. Auerbach *et al.* (48) found similar regional differences in tumor growth that might also be related to the capacity for vascularization at these sites. Dorsal injections also induced consistent responses, but the abdominal location was used almost exclusively in this study. Gels could be recovered intact by dissection of the underlying peritoneum (Fig. 2). The tissue in contact with the FGF- and heparin-supplemented gels contained abundant and readily visible blood vessels (Fig. 2). Large neovessels were also present on the surface (Fig. 3*A*, *B*), whereas small, tortuous vessels were observed within the gel (Fig. 3*C*). The effect of age of the animal on the angiogenic

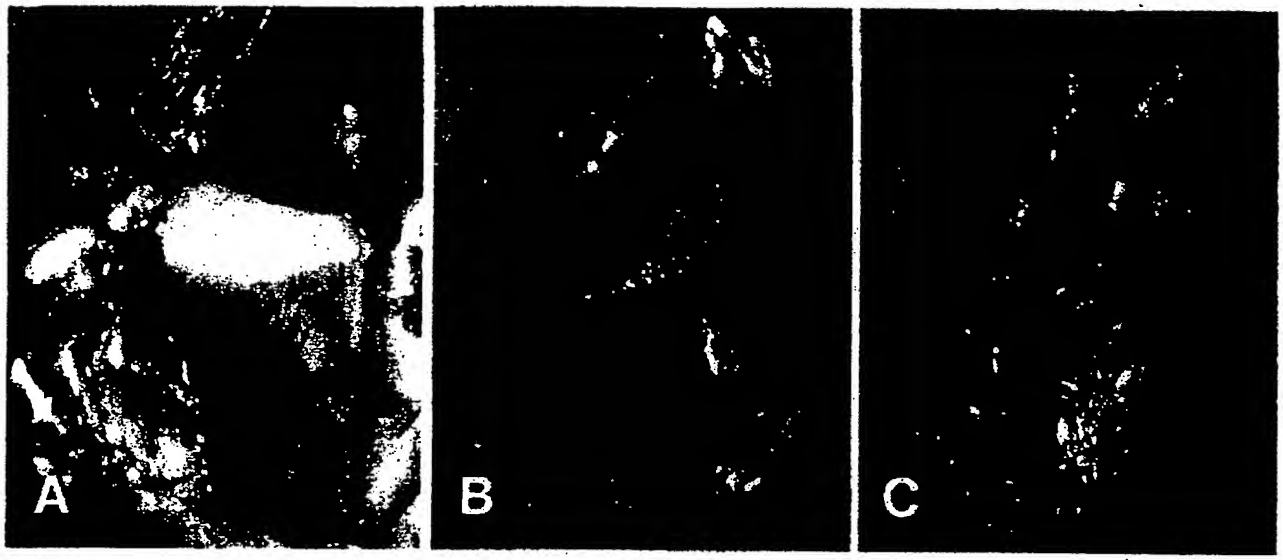

FIG. 1. Appearance of Matrigel gels on day 4 (*A*) or Matrigel supplemented with 1 ng/ml FGF and 40 units/ml heparin on day 1 (*B*) or day 4 (*C*) after subcutaneous injection. Overlying skin was removed to expose gels. Note that surface of gels as well as overlying skin flaps contain many vessels. Bleeding seen here was also seen with some Matrigel/FGF and Matrigel/heparin injections, but these produced little or no vessel infiltration and was <10% of the amount of hemoglobin found in Matrigel/FGF/heparin gels at 4 days (see also Table 1).

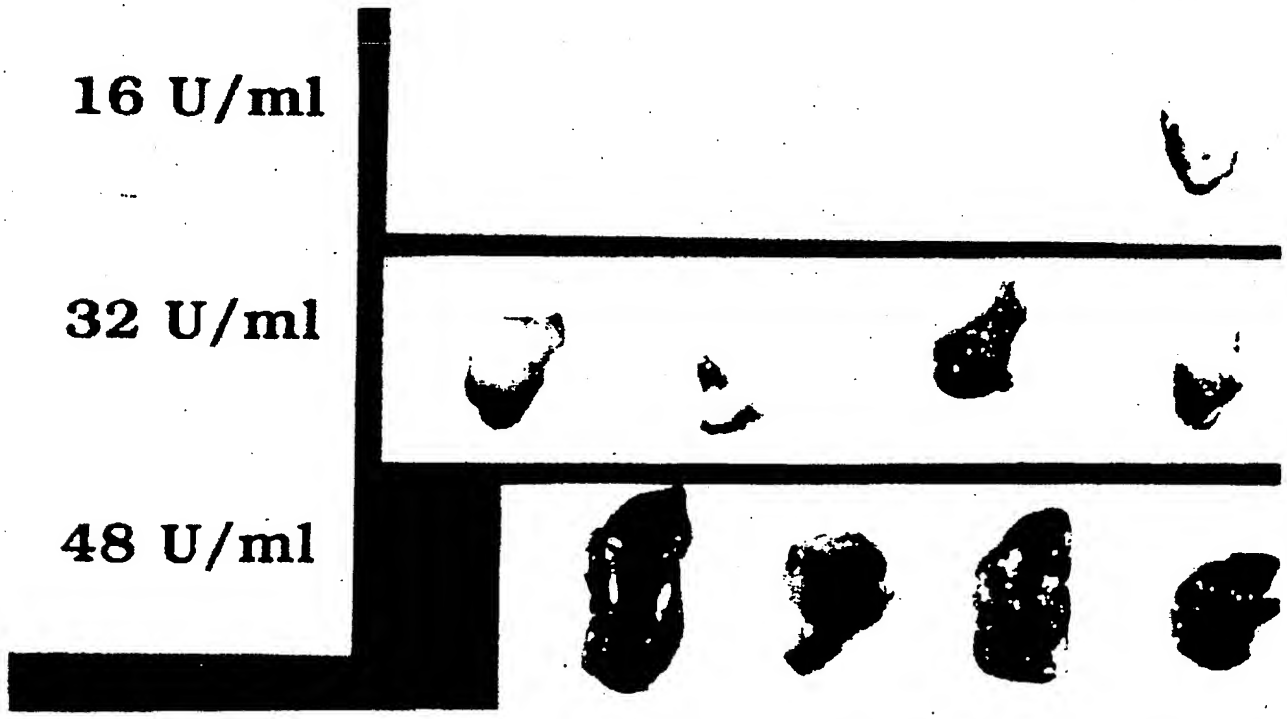

FIG. 2. Appearance of Matrigel gel recovered after 4 days *in vivo*: angiogenic response as a function of heparin concentration. Mice were injected with Matrigel and aFGF as in Figure 1*C* but with various heparin doses. After killing animals, skin was removed, and gels were cut out with intact peritoneal lining for support and placed on tissue culture dishes for photography. Each gel was between 0.8 and 1.4 cm long. Heparin dependence of response is apparent (see also Table 1).

response showed that vessel formation was reduced in young (6 month) animals compared with older mice (12, 18, or 24 months of age) where the response was typically twice as strong.

HISTOLOGY AND ENDOTHELIAL CELL STAINING

Sections examined with the Trichrome-Masson stain (Fig. 4) showed that cells invaded the gel within 24 hours and persisted for up to 8 days with a progressive increase

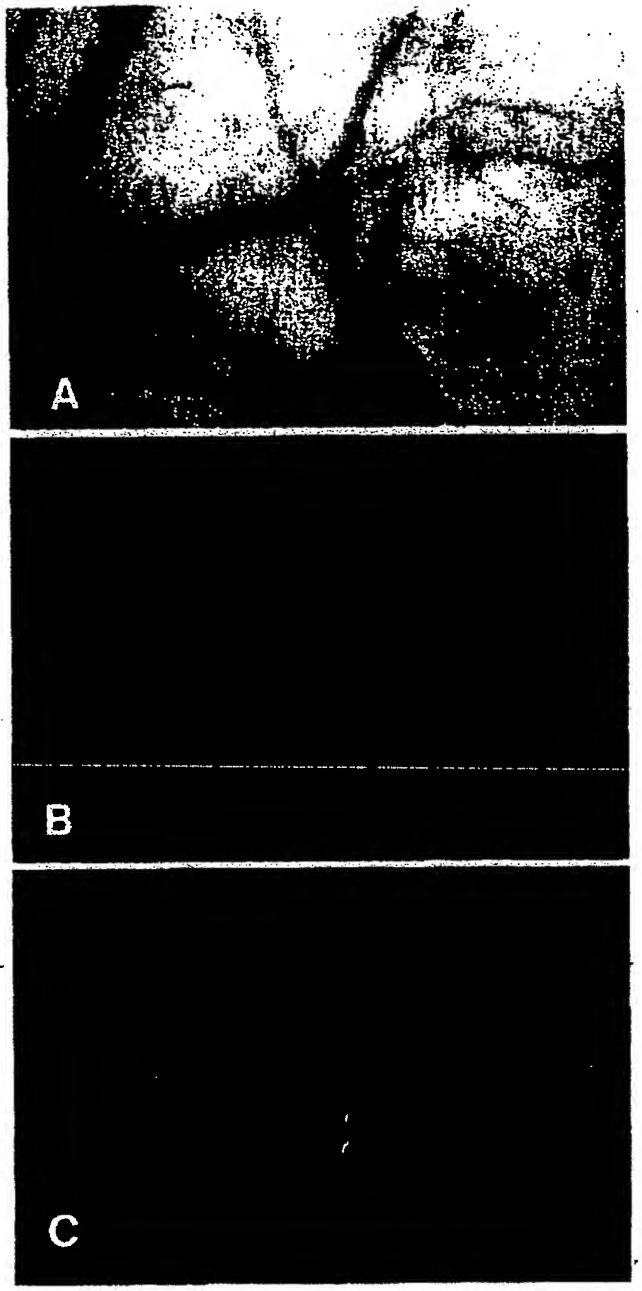

FIG. 3. Appearance of vessels associated with FGF/heparin-supplemented gels. Vessels surrounding gel appear to derive from peritoneal lining (*A*) and skin (*B*). *Arrows*, regions in injected gel that contain tortuous neovessels derived from ramified pre-existing vessels. Small tortuous tubes are also prevalent inside gel (*C*, *arrows*) that appears atypical.

in linear structures containing red blood cells which was indicative of functional vessels. Sections of the gel were reacted with antibody to factor VIII antigen (von Willebrand factor) to confirm the presence of endothelial cells in association with the vessels. The presence of capillary-sized vessels in the gel was apparent at 72 hours (Fig. 5). These neovessels were also apparent by 48 hours (not shown) and are smaller than other factor VIII positive structures (pre-existing vessels) on the periphery of the Matrigel (Fig. 5, arrowheads). Neovascularization was not observed at 24 hours, although inflammatory cells were observed in the region between the Matrigel and skeletal muscle.

QUANTITATION OF FGF-INDUCED ANGIOGENESIS

The increase in vessels in the gels, based on specific von Willebrand factor stain as quantitated by an image analysis system (Fig. 6), was similar to the increase in cells (hematoxylin/eosin and trichrome stain). Measurement of hemoglobin content indicated formation of a functional vasculature at the site of angiogenesis. As judged by hemoglobin content, the angiogenic response to FGF was time dependent, clearly visible by days 1–2, reached a plateau by days 3–4, and persisted through day 8 (Fig. 6, *lower panel*) occurring with similar kinetics as observed for the accumulation of neovessels (Fig. 6, *upper* and *middle panels*).

In the presence of heparin (64 units/ml), the maximal angiogenic response occurred at 1 ng/ml aFGF (Table 1) followed by a decrease and then a subsequent increase at higher levels of FGF. These data are consistent with the down-regulation of FGF receptors in the presence of higher levels of the growth factor (57). In some experiments, higher doses of FGF were used (125 and 250 ng FGF/ml), and these showed responses similar to those observed at 100 ng/ml. In contrast, heparin induced a linear increase in angiogenesis in the presence of 1 ng/ml FGF (Table 1). The lowest concentration of heparin that resulted in consistent vascularization of the gels was 40 units/ml. At this concentration of heparin, the FGF response was also biphasic with an optimum again at 1 ng/ml (data not shown). The amount of aFGF (0.5 ng) required for an angiogenic response in these assays is similar to the levels of FGF necessary for endothelial cell growth in culture (49, 50) and to the levels required to elicit an angiogenic response in the chick allantoic membrane (10). Our results suggest that the angiogenic response induced by heparin and aFGF occurs at physiologically relevant doses of FGF observed previously using other assays and other angiogenic factors.

INHIBITORS AND ACTIVATORS OF NEOVASCULARIZATION

We tested several cytokines for angiogenic activity in this assay in the presence of heparin to determine whether the assay was comparable to other established angiogenesis assays (Table 2). Of the various factors tested, aFGF, bFGF, and TNF-α induced an angiogenic response (Table 2), consistent with previous reports on these factors (1, 11, 51) whereas TGFβ, PDGF, interleukin (IL)-1, and IL-6 were inactive.

We also assessed the angiostatic activity of certain cytokines when included with aFGF (Table 3). IL-1β, IL-6 (52), and TGFβ inhibited the angiogenic response to aFGF. The TGFβ dose response showed inhibition at concentrations as low as 0.2 ng/ml. PDGF BB was also

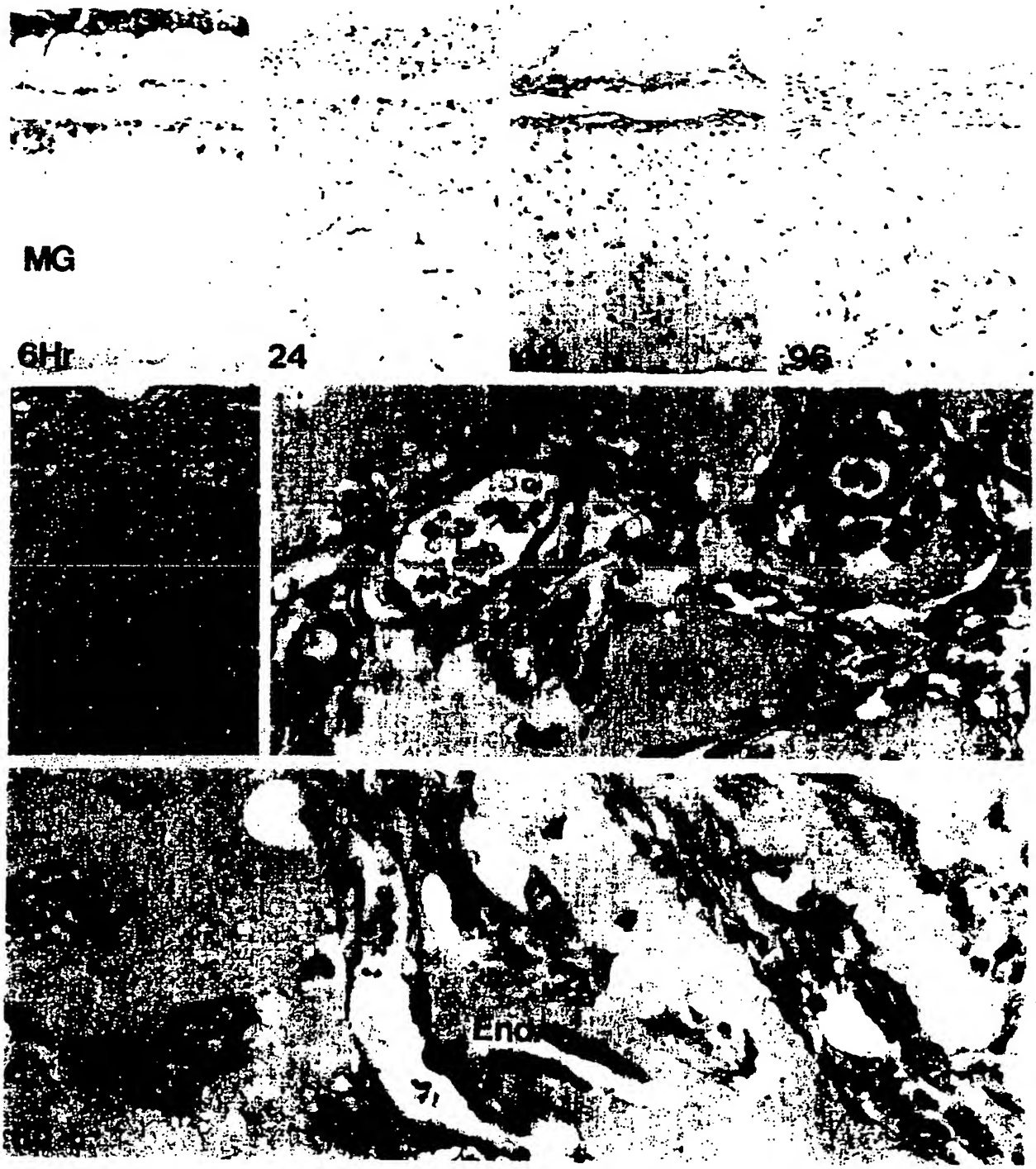

FIG. 4. Histological analysis of recovered gels: course of vessel formation. Samples were prepared for histology as described in "Methods" at different times after injection of Matrigel containing FGF and heparin. Trichrome-Masson stained specimens show the progressive invasion of cells into the Matrigel (MG) over 6, 24, 48, and 96 hours (top four panels; Mag = ×125). By 7 days, there is more organization of the cells into linear structures. At higher magnification (×500; middle panel, 7 day), the connective septa within the Matrigel exhibits large blood vessels from which an extension of a vessel into the Matrigel is evident (*two arrows*). At 8 days, many vessels within the Matrigel are well formed exhibiting a clear endothelium (End).

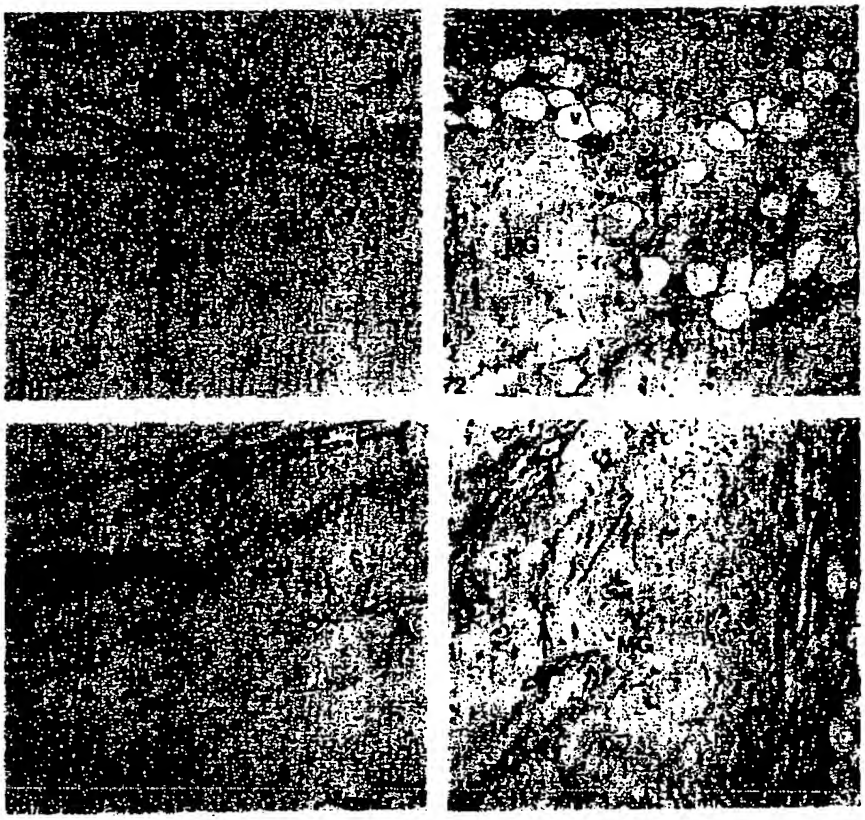

FIG. 5. Factor VIII staining of neovessels. Gels recovered after 1, 3, 4, and 7 days were stained with von Willebrand factor antibody as described in "Methods." Presence of neovessels (*arrows*) in Matrigel (MG) layer can be distinguished from existing vessels (*arrowheads*) near skeletal muscle (SM) and collagen (C) interface. Small vessels (72 hours), horizontally coursing structures (96 hours), and ramifying blood vessels (7 days) are noted. Vacuoles (v); ×40.

a potent inhibitor probably acting indirectly since endothelial cells do not express a receptor for this factor (53).

TIMP, a collagenase inhibitor (21), is also found in cartilage (22) where it may maintain cartilaginous tissue in an avascular state by inhibiting endothelial cell migration (22). Addition of recombinant 0.5 mg/ml TIMP in the Matrigel/heparin/FGF mixtures showed essentially complete inhibition of neovascularization at day 3 as measured both by hemoglobin content (Fig. 7) and by examination of the gel for infiltrating vessels (not shown). These observations are consistent with the known role of metalloproteases in the invasion of endothelial cells through basement membrane (43) and for the role of metalloproteases in angiogenesis (22).

DISCUSSION

We have developed a quantitative angiogenesis assay based on the ability of an extract of basement membrane proteins (Matrigel) to form a solid gel when injected into mice and to support a rapid and intense angiogenic reaction in the presence of FGF and heparin. Matrigel, while stimulating cell attachment and morphogenesis when used as a substratum in tissue culture, does not induce an angiogenic response *in vivo* alone. Matrigel has been found to promote the differentiation of endothelial cells into capillary-like structures in culture (12, 41) and when used as a vehicle *in vivo* may enhance the selectivity of endothelial cells entering the gel since basement membranes are not readily crossed by fibroblasts and certain other cells.

Gels supplemented with FGF and heparin induced intense vascularization. Numerous large vessels were apparent on the surface of the gel, whereas vessels within the gel were smaller and more tortuous. Vessel formation was quantitated by measuring the hemoglobin present in the dissected gels and confirmed by histological staining for von Willebrand factor and with Trichrome-Masson stain. Vessel formation was apparent as early as 2 days, reached a plateau after 4 days, and persisted up to 8 days. Maximal and consistent responses required both FGF and heparin, and distinct concentrations of each factor were required for optimal responses. The site of injection and age of the animal affected magnitude of the response.

The correlation of hemoglobin content with vessel formation was previously described using alginate-entrapped tumor cells to elicit angiogenesis *in vivo*. Factor VIII-stainable vessels were found to correlate with hemoglobin content and pooling of radiolabeled red blood cells at the alginate injection site (38). The requirement for heparin with FGF in angiogenesis assays (54) and fibroblast growth and differentiation (55, 56) appears to be due to both a stabilization of FGF and conformational changes in FGF required for receptor binding (57). Heparin also enhances the angiogenic activity of factors produced by 3T3 adipocytes (58), recently shown to be mediated by monobutyrin (16). In our assays, aFGF was

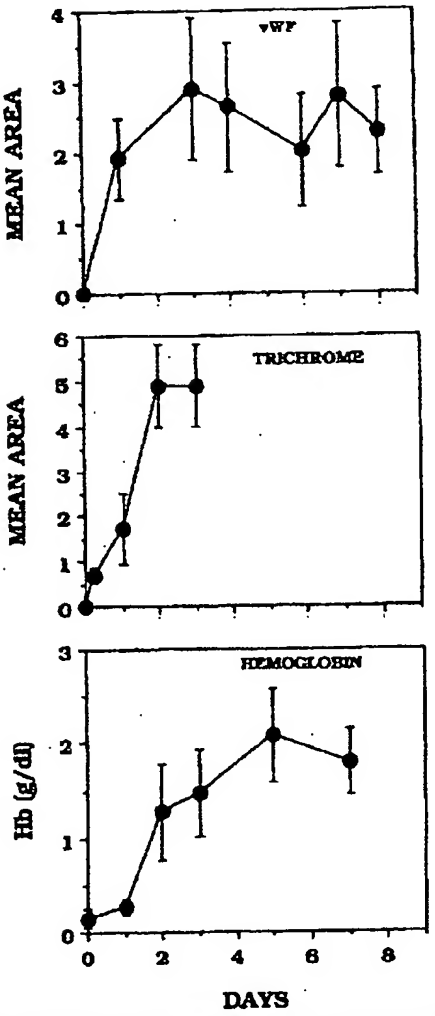

FIG. 6. Quantitation of neovascularization. Histological slides were examined with an Optomax image analysis system, and mean area in a 20× or 40× field was quantitated for slides stained with von Willebrand factor antibody (vWF) or Trichrome-Masson stain (Trichrome). Each point represents mean area per field ($\times 10^6$ μm^2) of 10–20 fields, and error bars are for standard deviation from mean. Hemoglobin measurements at these time points were determined as described in "Methods" and Table 1. Data represent the mean hemoglobin values from at least five mice per point with SEM as indicated.

TABLE 1. ACIDIC FGF DEMONSTRATES A BIPHASIC ANGIOGENIC RESPONSE THAT IS ENHANCED BY HEPARIN

	Hemoglobin[b]
	g/dl
aFGF (ng/ml)[c]	
0	0.28 ± 0.36
0.1	1.00 ± 0.45
1.0	3.20 ± 2.20
10	0.23 ± 0.15
100	0.94 ± 1.18
Heparin (units/ml)[d]	
2.0	ND[e]
6.0	0.04 ± 0.03
20	0.15 ± 0.20
32	0.16 ± 0.49
40[f]	0.69 ± 0.60
64	2.72 ± 1.00

[a] Matrigel gels contained 64 units heparin/ml and were processed 3 days after injection.
[b] Values (±SE) are averages of at least five animals.
[c] Matrigel contained FGF at 1 ng/ml for each experiment.
[d] ND, Not detectable.
[e] Heparin at 40 units/ml was the lowest concentration to yield consistent vessel formation.

TABLE 2. DETECTION OF ANGIOGENIC ACTIVITY USING VARIOUS NEOVASCULARIZATION FACTORS

Factors Added to Matrigel + Heparin	Hemoglobin
	g/dl
None	0.10 ± 0.02
aFGF (1 ng/ml)	1.30 ± 0.07[a]
TGFβ (20 ng/ml)	0.06 ± 0.02
PDGF BB	
2 ng/ml	0.10 ± 0.06
20 ng/ml	0.15 ± 0.08
200 ng/ml	0.07 ± 0.03
PDGF AB (6 units/ml)	0.11 ± 0.04
IL-1β (1 ng/ml)	0.22 ± 0.02
IL-6 (10 ng/ml)	0.18 ± 0.05
bFGF	
1.0 ng/ml	0.14 ± 0.13
10 ng/ml	0.20 ± 0.17[a]
100 ng/ml	0.86 ± 0.70
TNFα (10 ng/ml)	2.30 ± 2.00[a]

Matrigel (0.5 ml) and heparin (40 units/ml) were mixed with various factors and injected subcutaneously. Responses were quantitated 4 days later. TGF-β, PDGF BB, IL-6, IL-1β, or PDGF AB did not induce neovascularization.
[a] Acidic FGF, basic FGF, or TNF-α were potent inducers of angiogenesis.

potent at concentrations reported previously to be effective in both *in vivo* and *in vitro* assays. The course of the response was also comparable to results obtained with FGF in other assays and similar to that reported for other angiogenic agents like angiotropin (17). Heparin was required for angiogenesis in our assay even though heparan sulfate is present in the Matrigel possibly because the amount of heparan sulfate in the Matrigel is relatively low compared with normal basement membrane (59) and since FGF remains bound to heparan sulfate proteoglycan until released by enzymes (60). Not unexpectedly, the angiogenic response to FGF occurred more rapidly than the response observed with alginate-encapsulated tumor cells (38), which presumably require some time to generate their own factor(s). A related angiogenic factor, vascular permeability factor (13), has been shown to induce vascular permeability *in vivo* at 8 ng/animal and is active between 0.1 and 2 ng/ml as a mitogen for endothelial cells *in vitro*. In addition, the vascular permeability factor induces angiogenesis in the rat corneal assay at 20 ng (13). An unrelated chemical inducer of angiogenesis, monobutyrin (16), has been shown to be angiogenic in the chorioallantoic membrane

TABLE 3. INHIBITION OF ANGIOGENESIS BY PDGF, IL-1β, IL-6, AND TGF-β

Factors Added to Matrigel + Heparin + aFGF	Hemoglobin
	g/dl
None	1.30 ± 0.07
TNFα (10 ng/ml)	1.10 ± 1.10
TGFβ	
0.02 (ng/ml)	1.70 ± 1.50
0.2 (ng/ml)	0.08 ± 0.05
2.0 (ng/ml)	0.15 ± 0.13
20 (ng/ml)	0.24 ± 0.25
PDGF BB (200 ng/ml)	0.16 ± 0.07
IL-1β (1 ng/ml)	0.14 ± 0.10
IL-6 (10 ng/ml)	0.17 ± 0.12

Gels contained aFGF (1 ng/ml) + heparin (40 units/ml) and various cytokines. Hemoglobin levels in the gels are shown after 4 days. TNFα and TGF-β had no effect on the angiogenic response. PDGF BB, IL-1β, IL-6, and TGF-β inhibited the response.

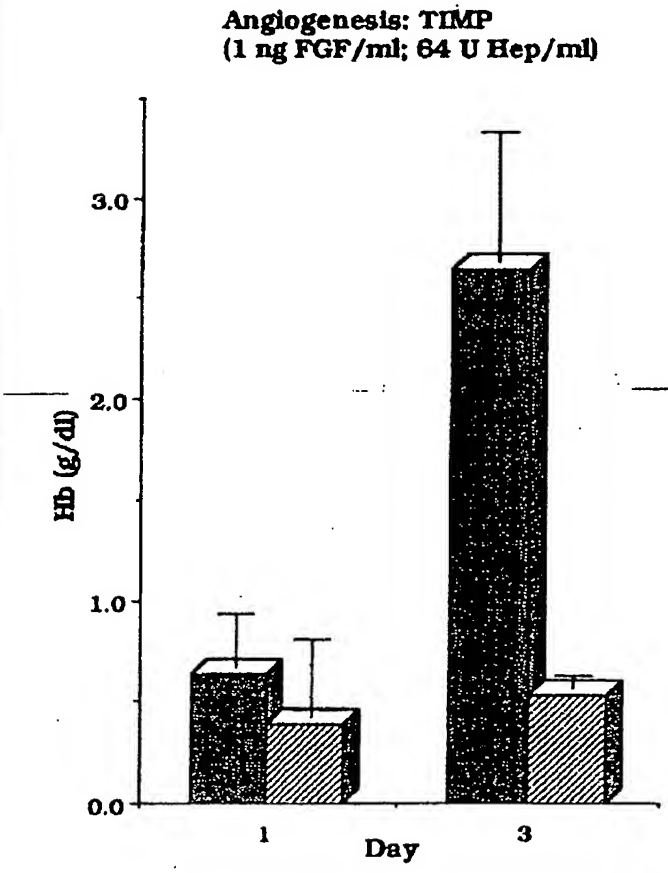

FIG. 7. Inhibition by TIMP: TIMP (collagenase inhibitor) is a potent inhibitor of aFGF-induced angiogenesis. Recombinant TIMP protein (0.5 mg/ml) was included with Matrigel (0.5 ml), FGF (1 ng/ml), and heparin (64 units/ml) at injection (*hatched bars*). Gels from at least five animals per point were analyzed after day 1 or 3. Shown for comparison are hemoglobin levels in gels that contained Matrigel, FGF, and heparin but lacked TIMP (*solid bars*).

assay at 20 pg (0.14 pmol), whereas aFGF in our assays is active at 0.025 pmol.

Other cytokines were tested in this assay including IL-1β, IL-6, and TGF-β and these were found to be potent inhibitors. IL-6 enhances production of TIMP (61), which may inhibit collagenase and endothelial cell migration (62). TGFβ inhibits endothelial cell proliferation and migration (63), although it exhibits angiogenesis *in vivo* in some assays (64). We have measured the content of TGFβ in the Matrigel to be 8–14 ng/ml dependent on batch. However, all TGFβ is in the latent form, and we cannot detect any active TGFβ in the preparations using the CCL64 mink lung epithelial cell bioassay that measures inhibition of proliferation of CCL64 cells by active TGFβ. Therefore the observed results with exogenous TGFβ (active form) reflect activity of the added factor. IL-1 has been shown to regulate endothelial cell growth via autocrine mechanisms (65) that may lead to programmed cell death (apoptosis) as is observed in endothelial cells deprived of FGF (66). TNF-α and bFGF induced neovessel formation. TNFα has been shown to activate macrophages (51) that in turn produce angiogenic factors.

In summary, the advantages of the assay presented here are that it is rapid, reproducible, quantitative, and does not require a surgical procedure for implantation. It allows detection of both angiogenic and anti-angiogenic factors and may allow isolation of those endothelial cells that penetrate into the gel. We have also used this system to assess the capacity of mice of different ages to initiate an angiogenic response, and this type of study would be of interest in both hypertensive and diabetic mice. Such systems may be useful in identifying and isolating biological factors and drugs able to regulate angiogenesis. In addition, the potential exists to induce an additional vascular supply in wounded or ischemic tissue where it is needed to restore normal healing and regeneration.

METHODS

ANIMALS, CELLS, AND GROWTH FACTORS

Female C57Bl/6 mice (Jackson Laboratories, Bar Harbor, Maine) were used at 6–8 weeks of age. Heparin was obtained from Gibco/Bethesda Research Laboratories. Bovine aFGF (HBGF I) bFGF (HBGF II) and TGF-β isolated from human platelets were from R & D Systems. Recombinant TNF-α was a generous gift from Dr. John Isaacs (Johns Hopkins University) and was originally obtained from Cetus Corporation. PDGF and recombinant IL-6, were from Collaborative Research (Bedford, Massachusetts). IL-1 was a kind gift from Dr. Nigel Waite at Upjohn. TIMP was a gift from Dr. David Carmichael (Synergen, Boulder, Colorado). CCL64 mink lung epithelial cells were obtained from American Type Culture Collection (Rockville, Maryland).

PREPARATION OF BASEMENT MEMBRANE MIXTURES

Reconstituted basement membrane (Matrigel) was prepared from the Engelbreth-Holm-Swarm tumor as described (45), sterilized by dialysis against chloroform, and stored at −20°C. Before use, Matrigel was thawed at 4°C and placed immediately on ice before addition of aFGF, heparin, or other growth factors.

Matrigel prepared by standard methods consists of 5–10 mg protein/ml and yields reproducible results in the angiogenesis assay. The commercial source of heparin (Gibco/Bethesda Research Laboratories) is critical and only batches yielding consistent results were used.

Histology and Factor VIII Related Antigen Staining

All specimens were fixed in 10% buffered Formalin for at least 24 hours, progressively dehydrated in increasing percentages of ethyl alcohol (70, 80, 95, 100, 100, and 100%), cleared in Histoclear, embedded in paraffin under vacuum, sectioned at 5 μm thickness, deparaffinized, and stained with Harris hematoxylin and eosin (67).

Selected specimens were also stained for Factor VIII-related antigen using an immunoperoxidase method (68) or Trichrome-Masson (69). Briefly, 5-μm sections were placed on silanized slides, dried overnight at 64°C, deparaffinized, hydrated, and placed into 3% hydrogen peroxide to quench endogenous peroxide. After rinsing in deionized water, the slides were enzymatically treated with 0.05% Pronase (Calbiochem, San Diego, California) in PBS with 0.114% EDTA at 37°C for 20 minutes. Enzyme activity was then abolished with 95% ethanol for 5 minutes. After PBS rinsing, rabbit anti-human von Willebrand Factor antibody (Dako, Carpinteria, California) diluted 1:1000 in 0.05% nonfat dry milk in PBS was applied to the slides that were placed in a humidity chamber overnight at 4°C. After rinsing in PBS the next morning, test slides were incubated at room temperature for 20 minutes in biotinylated antirabbit IgG (Vector, Burlingame, California) diluted 1:1000 in PBS with 0.5% nonfat dry milk. Nonimmune goat serum (5% v/v) was added to block nonspecific staining. Slides were then rinsed in three changes of PBS, incubated for 20 minutes in horseradish peroxidase conjugated streptavidin (Jackson ImmunoResearch, West Grove, Pennsylvania), diluted 1:1500 in PBS with 0.5% nonfat dry milk, rinsed in tap water, dried, mounted in Crystal Mount, dried at 80°C for 20 minutes, and coverslipped with Permount.

Image Analysis and Neovessel Quantitation

To measure the total area of neovessels, a computerized digitalyzer, the Optomax image analysis system (Optomax), was used. This system consists of a high sensitivity CCTV camera mounted on a Nikon Optiphot-2 microscope. The image is displayed on a color video monitor that is interfaced with a microprocessor. Histological slides stained with von Willebrand factor antibody or Trichrome-Masson stain were examined by adjusting the color contrast to enhance the specifically stained vessels. The mean area per field ($\times 10^5$ μm^2) from 10–20 fields (20× or 40×) was calculated with standard deviation from the mean. The vascularized area to be measured was chosen for its proximity to the skeletal muscle/collagen interface from which the neovessels originated before entering the Matrigel.

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Address reprint requests to: Antonino Passaniti, Gerontology Research Center, National Institute on Aging, 4940 Eastern Ave., Baltimore, MD 21224.

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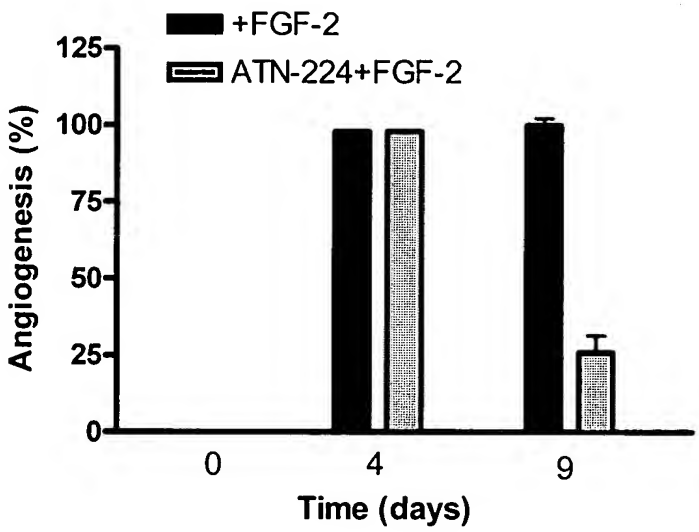
Exhibit 5

ATN-224 regresses established neo-vessels in a Matrigel Plug model of angiogenesis. Angiogenesis was stimulated using FGF-2. Treatment with ATN-224 (50 mg/kg by gavage every day for five days) was initiated on Day 5.



Combretastatin A-4 Phosphate Suppresses Development and Induces Regression of Choroidal Neovascularization

Hiroyuki Nambu,[1,2] *Rie Nambu,*[1,2] *Michele Melia,*[1] *and Peter A. Campochiaro*[1,2]

Purpose. Combretastatin A-4 (CA-4) is a naturally occurring agent that binds tubulin and causes necrosis and shrinkage of tumors by damaging their blood vessels. In this study the effect of a CA-4 prodrug, combretastatin A-4-phosphate (CA-4-P), was tested in two models of ocular neovascularization.

Methods. The effect of CA-4-P was quantitatively assessed in transgenic mice with overexpression of vascular endothelial growth factor in the retina (rho/VEGF mice) and mice with choroidal neovascularization (CNV) due to laser-induced rupture of Bruch's membrane.

Results. In rho/VEGF mice, daily intraperitoneal injections of 4.0 mg/kg CA-4-P starting at postnatal day (P)7, the time of onset of transgene expression, resulted in a significant reduction in the number of neovascular lesions and total area of neovascularization per retina at P21, compared with vehicle-injected mice. In mice with laser-induced rupture of Bruch's membrane, daily intraperitoneal injections of 75 or 100 mg/kg CA-4-P resulted in a significant reduction in the area of CNV at rupture sites compared with vehicle-injected mice. In mice with established CNV, daily intraperitoneal injections of 100 mg/kg CA-4-P for 1 week resulted in a significant reduction in CNV area at rupture sites compared with the baseline area before treatment or the area of CNV in vehicle-treated mice.

Conclusions. These data indicate that CA-4-P suppresses the development of VEGF-induced neovascularization in the retina and both blocks development and promotes regression of CNV. Therefore, CA-4-P shows potential for both prevention and treatment of ocular neovascularization. (*Invest Ophthalmol Vis Sci.* 2003;44:3650–3655) DOI:10.1167/iovs.02-0985

Tubulin-binding agents, such as vincristine, vinblastine, and colchicine, cause tumor necrosis from damage to tumor blood vessels, but at doses that are too toxic for patients to tolerate.[1,2] CA-4 is a naturally occurring structural analogue of colchicine that binds tubulin at the same site as colchicine, but with different characteristics[3,4] that impart selective toxicity to tumor vasculature.[5] Combretastatin A-4-phosphate (CA-4-P) is a more soluble, inactive prodrug that is converted to CA-4 by endogenous nonspecific phosphatases.[6] The selectivity of CA-4 for tumor vasculature allows for antitumor effects at doses of CA-4-P that are well tolerated.[7] A single dose of 100 mg/kg is well tolerated in adult mice and has beneficial effects on several types of tumors.[5,8–10] This finding led to a phase I study in patients with advanced cancer that showed a reasonable safety profile for 10-minute intravascular infusions of doses of 60 mg/m^2 or less, administered every 3 weeks, and some preliminary evidence of possible efficacy, in that a patient with anaplastic thyroid cancer had a complete response.[11] Therefore, CA-4-P shows considerable promise as a novel treatment for cancer.

Recently, it has been demonstrated that administration of CA-4-P results in microthrombus formation in new vessels surrounding a hyperplastic thyroid, indicating that its effects are not limited to tumor vasculature.[12] In neonatal mice with oxygen-induced ischemic retinopathy, daily intraperitoneal injections of CA-4-P starting before the onset of neovascularization suppressed the development of retinal neovascularization.[13] In this study, we sought to determine the effects of CA-4-P on subretinal and choroidal neovascularization.

Materials and Methods

Treatment of Rhodopsin/VEGF Transgenic Mice

Mice were treated in accordance with the recommendations of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. Hemizygous rhodopsin/VEGF transgenic mice[14,15] were given daily intraperitoneal injections of vehicle (n = 13 mice) or vehicle containing 2.2 (n = 5) or 4 mg/kg (n = 9) of CA-4-P (Oxigene, Inc., Boston, MA) between postnatal day (P)7 and P21. At P21, the mice were anesthetized and perfused with fluorescein-labeled dextran (2 × 10^6 average molecular weight, Sigma-Aldrich, St. Louis, MO), and retinal flatmounts were prepared as previously described.[15] Briefly, the eyes were removed and fixed for 1 hour in 10% phosphate-buffered formalin, and the cornea and lens were removed. The entire retina was carefully dissected from the eyecup, radially cut from the edge of the retina to the equator in all four quadrants, and flatmounted in aqueous mounting medium (Aquamount; BDH, Poole, UK) with photoreceptors facing upward. One retina of a mouse in the vehicle-treated group was severely damaged during dissection and could not be evaluated, leaving 25 retinas in the vehicle group for analysis. Flatmounts were examined by fluorescence microscopy (Axioskop; Zeiss, Thornwood, NY).

Quantitation of VEGF-Induced Neovascularization

Retinal flatmounts were examined by fluorescence microscopy at 400× magnification, which provides a narrow depth of field, so that when focusing on NV on the outer edge of the retina the remainder of the retinal vessels are out of focus, allowing easy delineation of the NV.[15] The outer edge of the retina, which corresponds to the subreti-

From the Departments of [1]Ophthalmology and [2]Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland.

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Corresponding author: Peter A. Campochiaro, Maumenee 719, Johns Hopkins University School of Medicine, 600 N. Wolfe Street, Baltimore, MD 21287-9277; pcampo@jhmi.edu.

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nal space in vivo, is easily identified and therefore there is standardization of focal plane from slide to slide. Images were digitized with a three-color charge-coupled device (CCD) video camera (IK-TU40A; Toshiba, Tokyo, Japan) and a frame grabber. Image analysis software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD) was set to recognize fluorescently stained neovascularization and used to delineate each of the lesions throughout the entire retina and calculate the number of lesions per retina, the area of each lesion, and the total area of neovascularization per retina.

Preventive Treatment of Laser-Induced CNV

Laser photocoagulation-induced rupture of Bruch's membrane was used to generate CNV.[16] Briefly, 4- to 5-week-old female C57BL/6J mice were anesthetized with ketamine hydrochloride (100 mg/kg body weight), and the pupils were dilated with 1% tropicamide. Three burns of 532 nm diode laser photocoagulation (75 μm spot size, 0.1 seconds duration, 120 mW) were delivered to each retina with the slit lamp delivery system of a photocoagulator (OcuLight GL; Iridex, Mountain View, CA) and a handheld coverslip used as a contact lens. Burns were performed in the 9, 12, and 3 o'clock positions of the posterior pole of the retina. Production of a bubble at the time of laser burn, which indicates rupture of Bruch's membrane, is an important factor in obtaining experimental CNV,[16] and therefore only burns in which a bubble was produced were included in the study. After laser burn, mice were treated with daily intraperitoneal injections of vehicle (group 1; $n = 41$) or vehicle containing 10 (group 2; $n = 10$), 20 (group 3; $n = 11$), 50 (group 4; $n = 11$), 75 (group 5; $n = 4$), or 100 (group 6; $n = 19$) mg/kg CA-4-P. After 2 weeks, mice were perfused with fluorescein-labeled dextran, and choroidal flatmounts were prepared as described for retinal flatmounts, except that the eyecup rather than the retina was cut with radial cuts and mounted. In each group, some eyes were unusable due to traumatic injuries from fighting among the mice or damage incurred during enucleation or dissection, resulting in the following number of eyes in each group: group 1, 76; group 2, 19; group 3, 21; group 4, 22; group 5, 8; and group 6, 37. Elimination of burns that had not ruptured Bruch's membrane, as indicated by the absence of a vaporization bubble, resulted in the following number of rupture sites for analysis in each group: group 1, 185; group 2, 41; group 3, 51; group 4, 58; group 5, 17; and group 6, 92.

Treatment of Established CNV

Adult female C57BL/6 mice ($n = 25$) had laser treatment of three locations in each eye, as described earlier. Only burns in which a bubble was produced were included. After 1 week, the mice were randomly divided into three groups: Eight mice were perfused with fluorescein-labeled dextran, and choroidal flatmounts were dissected to establish the baseline amount of CNV; eight mice were treated with daily intraperitoneal injections of vehicle; and nine mice were treated with daily intraperitoneal injections of 100 mg/kg CA-4-P. After 1 week of injections, the remaining 17 mice were perfused with fluorescein-labeled dextran and choroidal flatmounts were dissected. Elimination of burns that had not ruptured Bruch's membrane as indicated by absence of a vaporization bubble, resulted in the following number of rupture sites for analysis in each group: baseline, 42; vehicle, 31; and 100 mg/kg CA-4-P, 38.

Measurement of the Area of CNV

The area of CNV lesions was measured in choroidal flatmounts.[17] Flatmounts were examined by fluorescence microscopy, and images were digitized using a three-color CCD video camera and a frame grabber. Image-analysis software (Image-Pro Plus; Media Cybernetics) was used to measure the total area of hyperfluorescence associated with each burn, corresponding to the total fibrovascular scar.

Statistical Analysis

In mice with laser-induced rupture of Bruch's membrane, CNV areas were analyzed with a linear mixed model.[18] This model is analogous to analysis of variance (ANOVA), but allows analysis of all CNV area measurements from each mouse, rather than average CNV area per mouse, by accounting for correlation between measurements from the same mouse. The advantage of this model over ANOVA is that it accounts for differing precision in mouse-specific average measurements arising from a varying number of observations among mice. A log transformation was used on the area measurements before analysis so that they better met the normal distribution assumption of the analytic model. Probabilities for comparison of treatments were adjusted for multiple comparisons by the Dunnett method. $P \leq 0.05$ was considered statistically significant.

In rho/VEGF transgenic mice, the data for the number of neovascular lesions per retina, the average size of neovascular lesions, and the total area of neovascularization per retina were analyzed separately with a linear mixed model, as described earlier. A log transformation was applied to the number of lesions and the total area measurements before analysis, so that they better met the model assumption of normally distributed data. Probabilities for the comparison of treatments were adjusted for multiple comparisons using the Dunnett method. $P \leq 0.05$ was considered statistically significant.

Results

Effect of CA-4-P on the Development of Subretinal Neovascularization in Rho/VEGF Transgenic Mice

Litters of hemizygous rho/VEGF transgenic mice were divided into three groups and between P7 and P21 were given daily intraperitoneal injections of vehicle, or vehicle containing 2.2 or 4 mg/kg CA-4-P. At P21, mice treated with vehicle (Fig. 1A) or 2.2 mg/kg CA-4-P (Fig. 1B) showed numerous neovascular lesions (arrows). In contrast, fewer neovascular lesions were present in mice treated with 4.0 mg/kg CA-4-P (Fig. 1C, arrows). Image analysis confirmed that there were significantly fewer neovascular lesions and that each had a significantly smaller area than those in the retinas of vehicle-treated mice (Fig. 1D). There was no difference in number or area of lesions between vehicle-treated mice and mice treated with 2.2 mg/kg CA-4-P. The total area of neovascularization per retina was 0.01415 ± 0.00432 mm^2 in mice treated with 4 mg/kg CA-4-P, which was significantly less than that in mice treated with vehicle (0.06112 ± 0.03436 mm^2, $P = 0.0033$). The total area of neovascularization per retina was 0.04671 ± 0.01059 mm^2 in mice treated with 2.2 mg/kg CA-4-P, which was not significantly different from that in vehicle-treated mice ($P = 1.00$).

Effect of CA-4-P on the Development of CNV at Sites of Rupture of Bruch's Membrane

Adult mice tolerate much higher levels of CA-4-P than neonatal mice, which experience high mortality at doses above 5 mg/kg.[13] Because previous studies had demonstrated that single doses of 100 mg/kg are well-tolerated in adult mice,[5,8-10] we selected this for our highest dose in adults. Six of 25 mice treated with 100 mg/kg per day for 2 weeks died near the end of the treatment period, suggesting that this is at or near the maximum tolerated dose for a 2-week treatment period. There were no deaths in mice treated with 100 mg/kg per day for 1 week (see regression study described later), nor in mice treated with 75 mg/kg per day or lower doses for 2 weeks.

Treatment of adult C57BL/6 mice with 10, 20, or 50 mg/kg CA-4-P for 2 weeks after laser-induced rupture of Bruch's membrane resulted in CNV that had no significant difference in area compared with CNV in mice treated with vehicle (Fig. 2). Mice treated with 75 mg/kg CA-4-P had a moderate and statistically significant decrease in the area of CNV (Figs. 2E, 2G). Mice

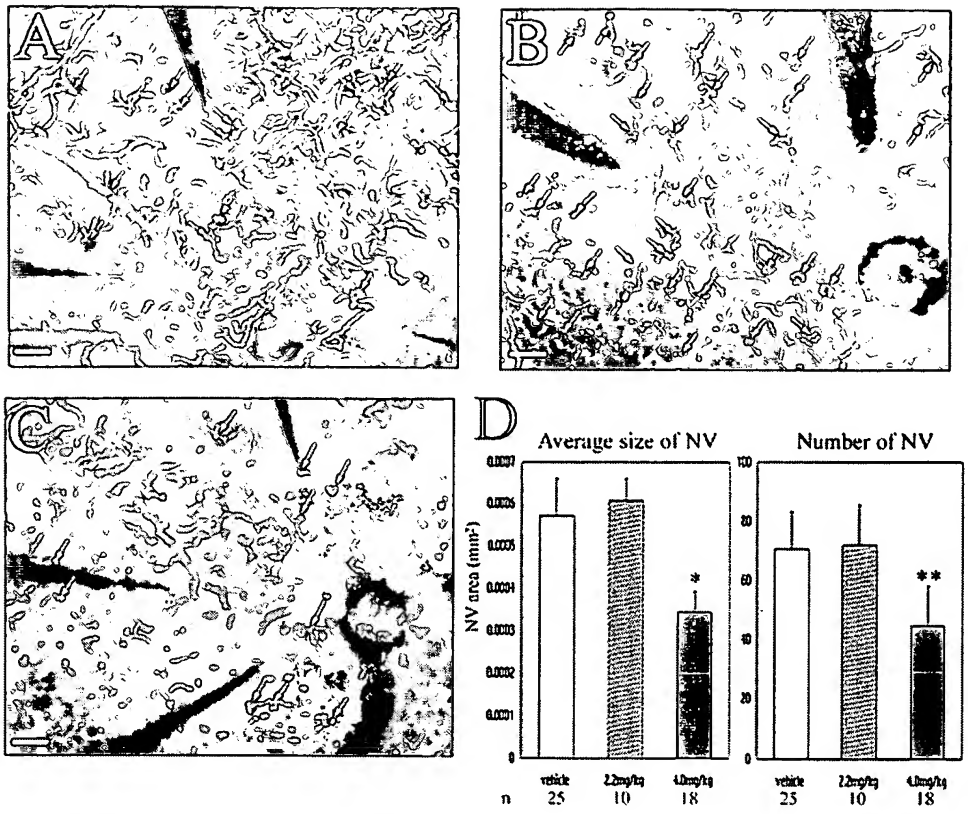

FIGURE 1. CA-4-P suppressed the development of subretinal neovascularization in transgenic mice that express vascular endothelial growth factor in photoreceptors (rho/VEGF mice). At P7, hemizygous rho/VEGF mice were started on daily intraperitoneal injections of vehicle or vehicle containing 2.2 or 4 mg/kg CA-4-P. At P21, mice were perfused with fluorescein-labeled dextran, and retinal flatmounts were prepared. Image-analysis software was used to compute the number and area of neovascular lesions and the total area of neovascularization on the outer surface of each retina. Retinas from mice treated with vehicle (A) or 2.2 mg/kg CA-4-P (B) showed numerous neovascular lesions (*arrows*), whereas mice treated with 4.0 mg/kg CA-4-P had fewer (C, *arrows*). Image analysis confirmed no difference between vehicle-treated mice and mice treated with 2.2 mg/kg CA-4-P, but mice treated with 4.0 mg/kg CA-4-P had significantly fewer neovascular lesions, and each had a significantly smaller area compared with those in the retinas of vehicle-treated mice (D). The number of retinas (n) evaluated for each group is shown. $^{*}P = 0.016$; $^{**}P = 0.035$ by linear mixed model adjusted for multiple comparisons by the Dunnett method. Bar, 100 μm.

treated with 100 mg/kg CA-4-P had a large, statistically significant decrease in CNV lesion size ($P < 0.0001$; Figs. 2F, 2G).

Effect of CA-4-P on Established CNV

To determine whether CA-4-P has any effect on already established CNV, mice were not treated for 1 week after laser-induced rupture of Bruch's membrane, and the baseline amount of CNV was measured (Fig. 3A). The remainder of the mice were treated with vehicle or vehicle containing 100 mg/kg CA-4-P, and after an additional week the amount of CNV at Bruch's membrane rupture sites was measured. There was no significant difference in size between baseline CNV lesions (Fig. 3A) and those in mice treated for the subsequent week with vehicle (Figs. 3B, 3D). However, mice treated for the subsequent week with 100 mg/kg CA-4-P had significantly smaller CNV lesions (Fig. 3C) than those at baseline (Fig. 3A) or those in vehicle-treated mice (Figs. 3B, 3D). This indicates that treatment with CA-4-P for 1 week resulted in partial involution of CNV.

DISCUSSION

In this study, we have demonstrated that a tubulin-binding agent, CA-4-P suppresses the development of subretinal neovascularization in rhodopsin/VEGF transgenic mice and suppresses the development of CNV at Bruch's membrane rupture sites. This suggests that when administered before onset of an angiogenic stimulus, CA-4-P can prevent these two forms of ocular neovascularization. A recent study has shown that CA-4-P can also prevent ischemia-induced retinal neovascularization in mice.[13] Therefore, CA-4-P joins a growing list of drugs that have potential as prophylactic agents for retinal and/or choroidal neovascularization.[19-30] However, CA-4-P also caused partial regression of established CNV, a finding that sets it apart from other drugs. To our knowledge, intraocular gene transfer of pigment epithelium-derived factor (PEDF) is the only other gene-therapy–based or drug treatment that has been shown conclusively to cause partial regression of CNV,[31] and therefore CA-4-P is in select company. Therefore, similar to PEDF gene transfer, treatment with CA-4-P has potential for treatment of established CNV.

There are many differences between neovascularization in tumors and CNV, and some agents such as interferon-α, which have been shown to inhibit some types of tumor angiogenesis,[32] do not inhibit CNV.[33] Therefore, it is hazardous to predict the effect of drugs on ocular neovascularization based on effects on tumor neovascularization. However, now that it has been demonstrated that CA-4-P causes regression of CNV, it is reasonable to consider findings in tumor models to formulate possible mechanisms by which CA-4-P may have this effect. Within 2 hours of a single intraperitoneal dose of 150 mg/kg CA-4 or 100 mg/kg CA-4-P, signs of hemorrhagic necrosis occur in murine tumors.[9] In vitro studies suggest that CA-4 binds to tubulin in endothelial cells, resulting in disruption of the cytoskeletal network, shape changes, and increased monolayer permeability.[5,9,34,35] Consequences in vivo include: increased vascular resistance[10,36]; vasoconstriction, which is partially ameliorated by nitric oxide (NO) and exacerbated by NO synthase (NOS) inhibitors[10,37]; increased vascular permeability[10,36,38,39]; platelet thrombi; and vascular shutdown.[36,39]

In cultured endothelial cells, CA-4-P causes shape changes and apoptosis of proliferating cells, but not of quiescent cells.[40] It appears that the cytoskeleton of newly formed cells is sensitive to CA-4-P, whereas the cytoskeleton of mature cells is not. This appears to underlie the preferential sensitivity of endothelial cells in tumor vessels, which unlike those in normal vessels, become thrombogenic, resulting in hemorrhagic necrosis of tumors.[5,8-10] The present study suggests that this differential sensitivity also applies to adult mice with CNV.

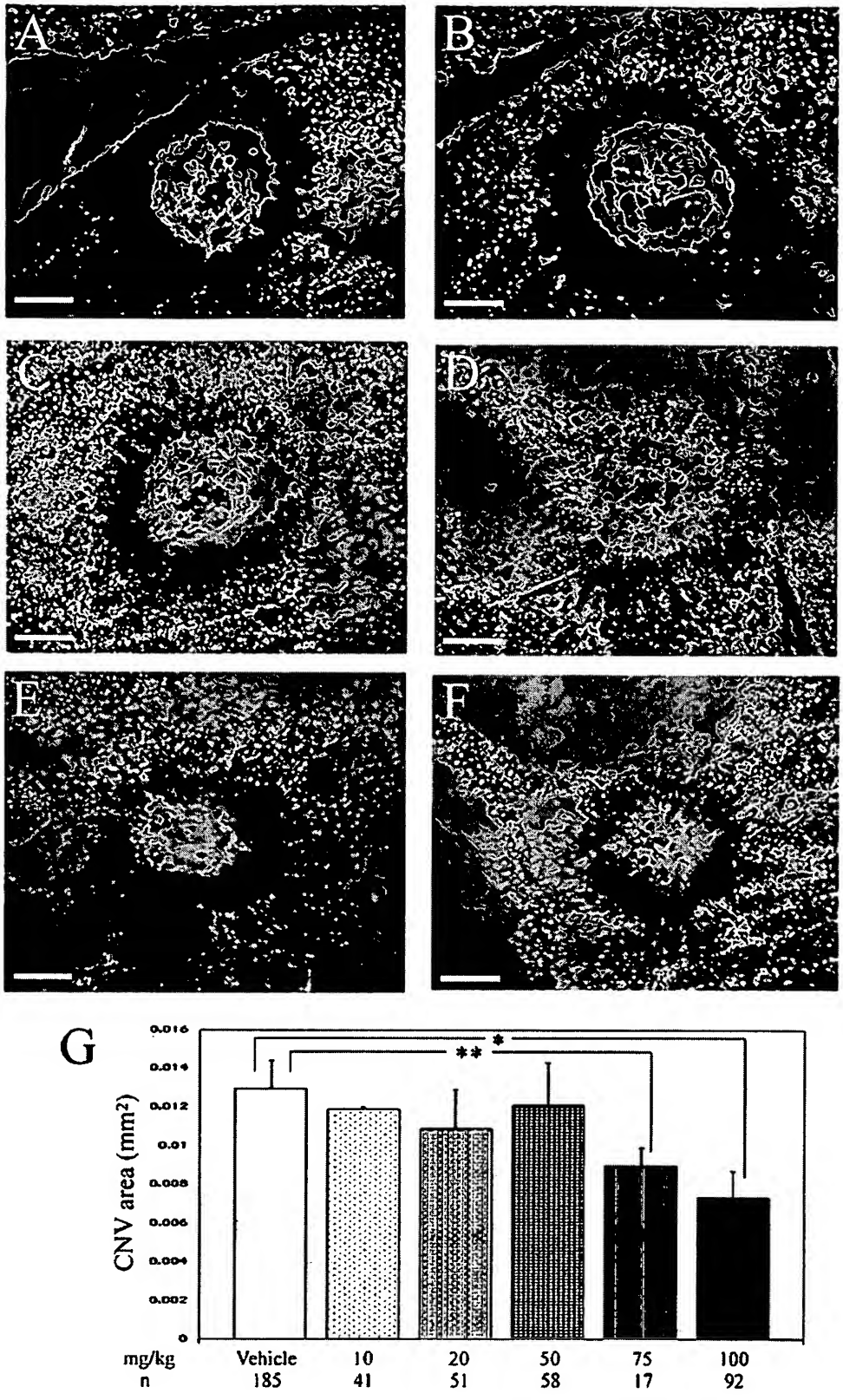

FIGURE 2. CA-4-P suppressed the development of CNV at sites of rupture of Bruch's membrane. Adult C57BL/6 mice had rupture of Bruch's membrane at three locations in each eye by laser photocoagulation and then were started on daily intraperitoneal injections of vehicle or vehicle containing 10, 20, 50, 75, or 100 mg/kg CA-4-P. After 2 weeks, mice were perfused with fluorescein-labeled dextran, and choroidal flatmounts were examined by fluorescence microscopy. Mice treated with vehicle (A), 10 (B), 20 (C), or 50 (D) mg/kg CA-4-P had similar amounts of CNV at rupture sites, with no statistically significant differences among these groups (G). Mice treated with 75 mg/kg had a moderate, statistically significant decrease in CNV lesion size (E, G). Mice treated with 100 mg/kg CA-4-P had a large, statistically significant decrease in CNV lesion size (F, G). The number of rupture sites (n) evaluated in each group is shown in (G). $^{*}P < 0.0001$; $^{**}P = 0.0069$ by linear mixed model adjusted for multiple comparisons by the Dunnett method. Bar, 100 μm.

We did not address toxicity of CA-4-P in our study, but our data suggest that a dose of 100 mg/kg per day is near the maximum tolerated dose in adult mice when given for 2 weeks, but is well tolerated for 1 week. Increasing the number or frequency of doses seems to increase toxicity. Doses as low as 25 mg/kg per day given every 12 hours cause severe damage to liver vasculature and death within 5 days.[13] A phase 1 trial in patients with advanced cancer demonstrated that an intravenous dose of 60 mg/m^2 or less every 3 weeks is safe and well tolerated, with some preliminary evidence of efficacy.[11] Clinical trials for ocular neovascularization in adult humans could be designed to take advantage of safety data for CA-4-P that is

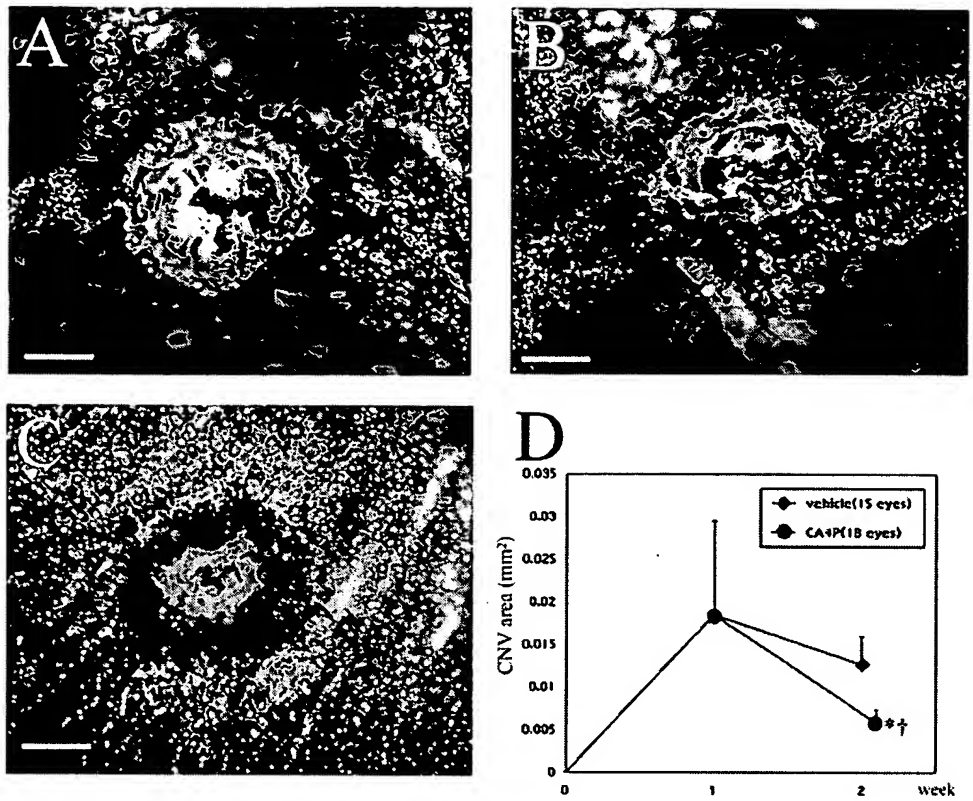

FIGURE 3. CA-4-P caused regression of established CNV. Adult C57BL/6 mice had rupture of Bruch's membrane at three locations in each eye by laser photocoagulation. After 1 week, some mice were perfused with fluorescein-labeled dextran, and choroidal flatmounts were examined by fluorescence microscopy to establish the baseline amount of CNV (A). The remainder of the mice were given daily intraperitoneal injections of vehicle (B) or vehicle containing 100 mg/kg CA-4-P (C). Measurement of CNV area by image analysis showed that mice treated with CA-4-P had significantly less CNV than mice treated with vehicle (D). The CNV area in CA-4-P-treated mice was also significantly less than that at baseline before treatment (D) indicating that CA-4-P caused regression of the CNV. There was no statistically significant difference between the baseline area of CNV and the area in mice treated with vehicle. The number of rupture sites evaluated in each group was: 1 week baseline, 42; 2 weeks vehicle-treated, 31; and 2 weeks CA-4-P-treated, 38. *P = 0.0003 by linear mixed model for comparison versus vehicle at 2 weeks; †P = 0.0002 by linear mixed model for comparison with baseline CNV area at 1 week. Probabilities are adjusted for multiple comparisons by the Dunnett method. Bar = 100 μm

being generated in oncology trials. Neonatal mice are particularly sensitive to the effects of CA-4-P. Mice survive a dose of 4 mg/kg between P7 and P21, which significantly suppresses VEGF-induced neovascularization in the retina, but doses above 5 mg/kg result in a high rate of mortality.[13] We postulate that cytoskeletal maturation occurs more slowly and to a lesser extent in neonates, making pathologic neovascularization exquisitely sensitive to CA-4-P, but other vessels throughout the body are only slightly less sensitive. This suggests that CA-4-P is not appropriate for treatment of retinopathy of prematurity or any other neovascular diseases in infants.

Although it is not certain that the mechanism by which CA-4-P causes partial regression of CNV is the same as the mechanism by which it affects tumor vessels, it is certainly a reasonable hypothesis and deserves investigation in future studies. It is also important to determine whether in treatment of CNV there is synergism between CA-4-P and hyperthermia, as is the case with treatment of tumors.[41] Similarly, it is important to investigate potential synergism between CA-4-P and photodynamic therapy. While gaining this information, it would be reasonable to consider a clinical trial based on safety data obtained in a phase I study in patients with advanced cancer.[11]

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Ocular Neovascularization: An Epidemiologic Review

PATRICIA LEE, MD,[1] CINDY C. WANG, MD, PhD,[1] AND ANTHONY P. ADAMIS, MD[1,2]

[1]Harvard Medical School, [2]Department of Ophthalmology, Massachusetts Eye and Ear Infirmary, and Surgical Research Laboratory, Children's Hospital, Boston, Massachusetts, USA

Abstract. Neovascularization occurs in many eye diseases, and its epidemiologic impact is significant. However, data on the prevalence and incidence of ocular neovascularization have never been compiled to demonstrate its pervasiveness. This overview of ocular angiogenesis provides a review of the epidemiologic literature for neovascularization in various parts of the eye, including the cornea, iris, retina, and choroid. Relevant disease states are reviewed, as are their risk factors, so that their pathogenesis can be better understood. Data on the prevalence and incidence of the major diseases involving angiogenesis are synthesized to provide statistical evidence of the span and magnitude of ocular neovascularization. These prevalence and incidence data on ocular neovascularization are extrapolated to USA population data where possible, and "worst-case" estimates are calculated as well. Information was gathered with a search of the MEDLINE database, published monographs and volumes, and consultation with a number of primary authors. This study attempts to unify much of past and present epidemiologic research, and the information is presented in sections divided according to the anatomy of the eye. (**Surv Ophthalmol 43**:245–269, 1998.

Key words. age-related macular degeneration • angiogenesis • diabetic retinopathy • epidemiology

During embryogenesis, endothelial cells differentiate from mesodermal blood islands and proliferate rapidly to form new blood vessels.[118,119] The vascular system develops through the mechanisms of vasculogenesis and angiogenesis. In vasculogenesis, blood vessels develop *de novo* from differentiating endothelial cells *in situ*, whereas in angiogenesis, capillaries originate from preexisting vessels. Vasculogenesis ceases after development, and endothelial cell proliferation nearly ceases in adults.[29] However, highly regulated angiogenesis does occur normally in adults and is responsible for physiologic functions, such as wound healing, ovulation, and placental maturation.[41] When unregulated, endothelial cells can cycle and divide abnormally to cause and contribute to pathologic states, such as tumor growth and eye disease.[118,119] In the eye this process is referred to as *ocular neovascularization.*

Neovascularization in the developed eye almost always impairs function. Schultze has stated that "neovascularization which accompanies some ocular diseases often appears to be misguided in its purpose and may ultimately lead to blindness.[129] These new vessels may grow into nearly all mature ocular tissue and affect the cornea, iris, retina, and optic disk.[59] Although no one factor can explain all causes of ocular neovascularization, multiple contributing factors have been implicated, such as inflammation and its molecular mediators, tumor angiogenic factors, and a hypoxic retinal diffusible factor.[59] The new ves-

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sels that form are structurally weak, both leaking fluid and lacking structural integrity. The resultant hemorrhage, exudates, and accompanying fibrosis often cause blindness.

Examples of the widespread nature of ocular neovascularization are presented in Table 1, in which prevalence and incidence data on neovascularization have been compiled and estimates of actual numbers of cases in the USA have been calculated. These calculations will be described throughout the text within each disease category. In some cases, the data allow only clear overestimates or worst-case estimates to be made. Following is a summary of the epidemiology of ocular neovascularization divided according to anatomic location.

I. Cornea

The cornea is normally avascular, but under certain conditions capillaries invade from the limbal vascular plexus. A wide variety of insults can cause many patterns of neovascularization, but they are generally grouped under three headings: superficial vascularization, vascular pannus, and deep stromal vascularization. In superficial vascularization, vessels sprout from the superficial marginal arcade and extend beneath the epithelium. Inciting insults include corneal trauma, mild chemical burns, inflammation, and infections.[36,59] In vascular pannus formation, collagen and vessels grow from the limbus onto the peripheral cornea. Pannus may form when an insult is sustained for a long period of time, and permanent scarring may result. Lastly, deep stromal vascularization can occur at any level of the stroma, from beneath Bowman layer to Descemet membrane. It is seen in conjunction with scleritis, serious anterior segment injuries, tuberculosis, and syphilis.[36,59]

The etiology of corneal neovascularization is not completely understood, and much of the present data are derived from animal models.[4,90] When the cornea is injured within the limbal margins, epithelial defects are healed by corneal epithelium and adjacent limbal epithelium. The latter contains stem cells that differentiate into normal corneal epithelium. Defects that result in the destruction of the limbal stem cells are healed by conjunctival epithelium. The conjunctival epithelium contains goblet cells and is vascularized. This epithelial phenotype is optically inferior and leads to degradation of the visual image. In addition, this type of defective epithelium, which is often associated with chemical burns and Stevens-Johnson syndrome, interferes with corneal function and results in recurrent corneal erosions, decreased vision from an irregular optical surface, weakened tensile strength, and incompetent barrier function.[63] Interestingly, selective destruction of corneal vessels in animal models can cause the abnormal corneal epithelium to transdifferentiate and take on many of the phenotypic characteristics of normal corneal epithelium. Thus, it appears that the presence of corneal vessels can affect the corneal epithelial phenotype.[63] Moreover, these data suggest that antiangiogenic therapy may be able to induce a defective epithelium to transdifferentiate into a normal cornea-like epithelium.

A wide variety of diseases are known to manifest corneal neovascularization (Table 2).[90] Yet, no study exists that illustrates the incidence and prevalence of corneal neovascularization in the general population. However, in a population of patients presenting for an eye examination, corneal neovascularization was found in 35 (4.14%) of 845 consecutive patients who came to the General Eye Service of the Massachusetts Eye and Ear Infirmary in 1996.[22] In 4 of these patients (12%), the neovascularization was associated with a decrease in visual acuity. In this patient population, corneal neovascularization was associated with the following conditions: contact lens wear (14/35 cases), eyelid inflammation (7/35), prior surgery (5/35), anterior segment neovascularization (2/35), trauma (1/35), trichiasis (1/35), and herpes infection (1/35). In 4 of 35 cases, no identifiable cause could be established. As estimated by the National Society to Prevent Blindness, there were 33.7 million office visits for eye care (all reasons) in the USA in 1976.[10] Applying the 4.14% prevalence to this figure, we can make a worst-case estimate that for a given year, 1.4 million people in the USA may have corneal neovascularization (Table 1). This figure is a clear overestimate, as the 33.7 million office visits include repeat eye care visits unlike the above study. Also, patients presenting for eye examinations are self-selected and may give a falsely higher prevalence rate of corneal neovascularization than is found in the general population. Population-based studies are needed to provide more accurate estimates of the scope of this problem. As illustrated in this study, corneal neovascularization complicates a number of primary corneal conditions. In a subset of cases, corneal neovascularization can lead to vision loss.

A. INFECTION

Although no studies specifically document the incidence and prevalence of neovascularization in ophthalmic infections, data are available on the prevalence of eye disease and blindness as a result of these infections. As neovascularization is frequently a sequela of ocular infection, the extent of blindness from infection paints a general picture of

the extent of corneal neovascularization that occurs worldwide.

Trachoma, a chronic infection of the conjunctiva caused by the microorganism *Chlamydia trachomatis*, is the leading cause of preventable blindness in the world.[105,140] This disease, seen mostly in northern Africa, the Middle East, India, and southeast Asia, is responsible for at least 6 million cases of blindness worldwide, according to World Health Organization reports in 1970; almost all of these cases of blindness involve corneal neovascularization and fibrous pannus formation.[1,161] Trachoma is much less prevalent in the USA, representing only 0.2% of all legal blindness cases, and none of these cases was reported in people less than 45 years old in 1980.[10] Because an estimated 900,000 Americans older than 40 years were legally blind in 1995, there are approximately 1,800 Americans (0.2%) with blindness from trachoma with pannus and neovascular scarring (Table 1).[146]

Onchocerciasis, a disease involving the filarial parasite, *Onchocerca volvulus*, is another significant cause of blindness related to corneal neovascularization. With time, a fibrovascular pannus develops, and ultimately, the whole cornea becomes opaque and neovascularized.[144] In equatorial Africa and areas of Central and South America, up to 50 million people are estimated to be infected, and 1 million people consequently lose their vision. In hyperendemic areas, almost every person is infected; 10% of the total population and half of those older than 40 years are blind from this disease.[144] In the western hemisphere, herpes simplex keratitis is the most common infectious cause of corneal blindness. In the USA alone, 500,000 cases of ocular herpes simplex virus are reported, and 1 in 10,000 infants born is infected with neonatal herpes simplex virus.[151] The recurrent form of this disease, which usually affects the stroma, is most often associated with neovascularization; however, its incidence and prevalence are not known.

B. CONTACT LENS WEAR

Contact lens-induced corneal neovascularization has come to attention in the past 2 decades because of its increasing prevalence. Prevalence is extremely low in patients wearing hard lenses. Corneal neovascularization is more prevalent when soft hydrogel lenses are worn, especially on an extended-wear basis. Efron compiled several prevalence studies of contact lens-induced neovascularization.[36]

The prevalence of corneal neovascularization was negligible for hard gas-permeable lenses in both daily wear (0.0%) and extended-wear (0.0%) usage.[71,97] Soft hydrogel lenses used for daily wear were associated with a higher prevalence rate of 1.3%.[122] Meanwhile, prevalence reports for extended-wear usage of hydrogel lenses for cosmetic purposes were variable. A retrospective study by Lamer[95] reported a prevalence rate of 0.2% in 400 patients using extended-wear soft lenses for more than 4 years. Retrospective studies are more likely to underestimate a sometimes subtle sign, such as corneal neovascularization, and often report lower prevalence rates than prospective studies. Stark and Martin, in a prospective study, noted neovascularization in 18 (8.7%) of 207 of lens-wearing eyes in which extended-wear soft lenses had been worn from 4 to 8 years.[138] In another prospective study, Binder similarly found corneal neovascularization in 7.0% of 1,099 myopic patients after 38 months of using extended-wear soft lenses.[8] This study also reported that the incidence increased with duration of lens wear.

The prevalence of neovascularization seen with extended-wear soft lenses used in aphakic patients was also reviewed. In a study by Spoor et al, superficial vascularization was noted in 14.2% of 140 aphakic eyes in patients wearing lenses for 1 to 37 months.[137] The authors reason that the high prevalence is not surprising in view of the trauma that the cornea endures from surgery.

Studies have also addressed the prevalence of corneal neovascularization with therapeutic usage of hydrogel lenses. Schecter et al studied 115 patients with contact lenses for treatment of various corneal diseases, and 90% of these patients were wearing their lenses continuously.[127] Corneal neovascularization was demonstrated in 35% of this group, and the authors found no relationship between duration of lens wear and the prevalence of neovascularization. They concluded that the extent of vascular response may have been related more to the underlying corneal disease being treated than to the duration of lens wear.[127]

In 1989 it was estimated that more than 13 million people in the USA wore soft contact lenses for refractive correction; of that group, 4 million used extended-wear soft lenses, and 9 million used daily-wear soft lenses.[128] Among daily-wear soft lens users, 1.3% or an estimated 120,000 of 9 million people, had corneal neovascularization.[122] For extended-wear usage of soft lenses, the prevalence ranged from 0.2% to 8.7% in the studies cited above.[8,95,138] Of the 4 million people using extended-wear soft lenses in 1989, we can calculate that 8,000 (0.2%) to 350,000 (8.7%) of them had new corneal vessels. Thus, the total number of people in the USA with contact lens–induced neovascularization is calculated to be 128,000 to 470,000, based on 1989 estimates of contact lens usage (Table 1). Since then, the use of daily-wear disposable contact lenses has increased dramatically, and disposable lenses may make an enormous difference in the incidence of corneal

TABLE 1

Estimates of the Prevalence and Incidence of Ocular Neovascularization in the USA

Condition	Reported Prevalence of NV	Reported Incidence of NV	Estimated Prevalence of NV	Estimated Incidence of NV (per year)	Relationship of NV to Vision Loss
Cornea					
All forms of NV	4.14% of patients of general eye service			<1.4 million[*]	Low
Contact lens wear	0.2-8.7% using soft extended-wear and 1.3% using soft daily-wear lenses		128,000–470,000[†]		Low
Alkali and other chemical burns				<37,000[‡]	High
Trachoma	0.2% of total cases of legal blindness		1,800 blind with fibroneovascular-scarring[§]		Medium
Iris					
All NV requiring enucleation	12-15% of enucleated eyes			1,320–1,650[‖]	High
Retinoblastoma	44% of enucleated eyes with retinoblastoma			<1,540[¶]	Low
CRVO	20% of CRVO cases			3,800[#]	Medium
Diabetes mellitus	2.3-2.5% of proliferative diabetic retinopathy cases		>16,100–17,500[**]		Medium
Retina					
Proliferative diabetic retinopathy	23% of younger-onset diabetics; older-onset:10% (insulin users), 3% (no insulin)	10-year incidence of 30% in younger-onset diabetics; older onset: 24% (insulin users),10% (no insulin)	>700,000 >91,500[††]	High	
All forms of diabetic retinal NV, including IRMA	40% of younger-onset diabetics; older-onset: 30%(insulin users), 10% (no insulin),1.7% of all Americans (aged 43–84 years)		>1.5–2.1 million[§§]		High
Retinopathy of prematurity	12-29% of premature newborns (28–31 wk); 2–20% (32–35 wk); 0–3.5% (32–35wk)		180,000[‖‖]	High	
Sickle cell retinopathy	20% of sickle cell anemia adults		5,500[¶¶]		Medium
	40% of hemoglobin SC disease adults		10,200[##]		
Choroid					
Age-related macular degeneration	(Refer to Table 6 for breakdown of data)	600,000-1.77 million	60,000–110,000	High	

CRVO = central retinal vein occlusion; IRMA = intraretinal microvascular abnormalities; NV = neovascularization; NVI = neovascularization of the iris; PDR = proliferative diabetic retinopathy.

[*](4.14% in 1996)(33.7 million office visits in USA for eye care per year in 1976) = 1.4 million new cases of corneal neovascularization per year.[10,22]

[†](1.3%)(9 million daily soft-lens wearers in USA in 1989 estimate) = 120,000.[122,128] (0.2% to 8.7%)(4 million extended-usage soft-lens wearers in USA in 1989 estimate) = 8,000 to 350,000.[8,95,128,138] Total = (120,000 + 8,000) to (120,000 + 350,000) = 128,000 to 470,000 Americans with corneal neovascularization related to contact lens wear.

[‡]37,000 eye injuries per year have been reported in the USA from all chemicals (alkali, acids, dyes, etc.) in 1977.[10] Thus, a worst-case estimate for cases of neovascularization from alkali and other chemical burns in the USA is 37,000/year.

[§](0.2% in 1980)(900,000 legally blind Americans over age 40 years in 1995 estimate) = 1,800 blind with fibroneovascular scarring from trachoma.[10,146]

[‖] (12% to 15%)(11,000 enucleations per year in USA in 1976) = 1,320 to 1,650 cases of NVI in enucleated eyes per year.[5,10,129] This may be a low estimate because the reported figure of total enucleations/year in the USA was based on 1976 data.[10]

[¶]44% of eyes enucleated secondary to retinoblastoma were reported in 1968 to have NVI.[156] During 1974–1985, the incidence of retinoblastoma in the USA was 1 in 20,000 live births.[142] An overestimate of the incidence of NVI related to retinoblastoma = (44%) (1/20,000 live births)(69.5 million single live births in USA per year during 1982–1988) = 1,540 new cases of NVI related to retinoblastoma per year.[18,142,156]

[#]NVI was reported in 1979 in 20% of CRVO cases.[134] Four-year incidence of CRVO = 0.72 per 1,000 in a general population over age 40 years (from 1988 estimate).[27] (20%)(0.72/1,000)[1/4 years](106 million Americans over age 40 years in 1995) = 3,800 new cases of NVI related to CRVO in Americans over age 40 years per year.[27,134,148]

neovascularization. Currently, no studies have been done on disposable contact lenses and corneal neovascularization.

The cause of contact lens-induced neovascularization is not completely understood, although there appears to be a strong association with corneal hypoxia. Neovascularization caused by contact lens wear is generally mild when encountered and does not affect vision in the aggressive manner that infections do. However, it is a sign of corneal distress and therefore deserving of attention.

C. ALKALI AND OTHER CHEMICAL BURNS

The pH of alkaline substances allows them to damage and penetrate the cornea and anterior chamber extremely rapidly. This type of trauma is typically worse than exposure to acids or other solvents. Neovascularization is part of the repair of extensive damage caused by alkaline chemicals. It may become pathologic, however, in the late reparative phase that occurs after severe burns. In this phase, epithelialization is complete, but a fibrovascular pannus may overgrow the cornea because of destruction of the limbal epithelial stem cells. Alternatively, reepithelialization may progress too slowly or not at all. The ocular tissue can heal with severe neovascularization, and even when it is healed, a fibrovascular pannus may remain. In this overly vascularized and vulnerable state, the cornea has a propensity to ulcerate in response to any subsequent insult.[7] Because no prevalence studies have been done on this topic, we can provide an upper-end estimate of neovascularization caused by alkaline substances. Based on estimates from the United States Consumer Product Safety Commission, 2,632 cases of eye injuries from ammonia and detergents (alkaline substances) were reported in 1977.[10] The eye injuries from all types of chemicals (alkali, acids, dyes, varnish removers, etc.) totaled 37,011 cases. Thus, a worst-case estimate for the number of cases per year of neovascularization in the USA from alkali and other chemical burns is approximately 37,000 (Table 1).

D. SUMMARY

Corneal neovascularization has been reported in 4.14% of patients presenting for general ophthalmologic care in the USA, representing an estimated 1.4 million Americans.[22] Worldwide, it is associated with devastating infections, which cause millions to lose their sight. Specifically, chlamydial infections affect 400 million and blind 6 million.[161] Onchocerciasis infections affect 50 million and blind 1 million.[144] Herpes simplex eye infections are reported in 500,000 cases in the USA alone.[151] Neovascularization of the cornea is also notable in extended-wear usage of hydrogel contact lenses. The prevalence of neovascu-

**(2.3% to 2.5%) (700,000 Americans with proliferative diabetic retinopathy in 1980–1982) = 16,100 to 17,500 Americans with NVI related to diabetes.[33,84] This is a low estimate because the Diabetic Retinopathy Study prevalence data on diabetic NVI are much lower than data from other studies. Also, these low prevalence figures were applied to the estimate by Klein et al of only 700,000 Americans with PDR based on 6 million known diabetic Americans in 1980–1982.[84] It is currently reported that there are more than 14 million Americans with diabetes.[79]

††This estimate of 700,000 Americans with PDR was made by Klein et al based on their prevalence findings, 1980–1982 population data, and 6 million Americans known to have diabetes.[81,86,87] This is a low estimate for today's population because it is currently reported that there are more than 14 million Americans with diabetes.[79]

‡‡An estimated incidence of 915,000 new cases of PDR over 10 years (or roughly 91,500 per year) was calculated by Klein et al based on their incidence findings and 6 million Americans with known diabetes.[85] This is a low estimate because it is currently reported that there are more than 14 million Americans with diabetes.[79]

§§From a retabulation of the prevalence data from the Klein et al study to include IRMA, the prevalence of all forms of retinal neovascularization is roughly three times the Klein et al reported prevalence of proliferative diabetic retinopathy.[86,87] Klein et al applied their original data to estimate that 700,000 Americans have PDR based on 6 million known diabetic Americans. Thus, (3) × (700,000) = 2.1 million Americans have all forms of diabetic retinal neovascularization, including IRMA. As described above, this may also be a low estimate because there are currently more than 14 million diabetic Americans.[79] Another study (1988–1990) showed that 1.7% of a general population (aged 43–84 years) had diabetic retinal neovascularization, including IRMA.78(1.7%)(90.2 million Americans in 1995 between the ages of 43 and 84 years) = 1.5 million Americans with all forms of diabetic retinal neovascularization, including IRMA.[78,148]

¶¶From 1988 data, a reported 5.1 million low-birth-weight (defined as < 2,500 g) infants are born per year in the USA.[18] Most of the infants who weigh 2,500 g or less at birth are the older premature newborns (32-35 weeks of gestation). The prevalence of retinal neovascularization for 32-35-week-gestation premature infants has been reported to range from 0% to 3.5%.[12,70,73,110,112,113,117,125,162,163] Thus, an upper estimate of the incidence of retinal neovascularization secondary to retinopathy of prematurity is (3.5%)(5.1 million/year) = 180,000 new cases per year.

##(20% of sickle cell anemia adults) (0.14% prevalence of sickle cell anemia in African-Americans reported in 1981) (19.7 million African-Americans aged 18–64 years in 1995) = 5,500 sickle-cell anemia African-Americans with neovascular sickle cell retinopathy.[25,42,148,160]

***(40% of hemoglobin SC disease adults) (0.13% prevalence of hemoglobin SC disease in African-Americans reported in 1981) (19.7 million African-Americans aged 18–64 years in 1995) = 10,200 hemoglobin SC disese African-Americans with neovascular sickle cell retinopathy.[25,42,148,160]

TABLE 2

Diseases Associated With Corneal Neovascularization

Contact lens wear*
Trauma and prior surgery*
Infections
Bacteria and other microorganisms, e.g., *Chlamydia, Staphylococcus, Streptococcus, Pseudomonas*
Viruses,* e.g. herpes simples, herpes zoster
Protozoa, e.g., *Onchocerca volvulus, Leishmania brasiliensis*
Alkali burns
Immunologic diseases
Stevens-Johnson syndrome
Graft rejection
Cicatricial pemphigoid
Degenerative disorders, e.g., pterygium, Terrien's marginal degeneration

*Most frequently associated with corneal neovascularization in the United States.

Modified from Klintworth.[90]

larization ranges from 125,000 to 470,000 people in the USA who wear soft lenses for refractive correction. All of these data indicate that corneal neovascularization is a significant contributor to eye disease.

II. Iris

Neovascularization of the iris is a frequent cause of blindness and enucleation. It almost never starts as a primary condition but instead develops secondary to disease elsewhere in the eye. It occurs as part of a wide variety of ocular and systemic disorders, such as diabetes and central retinal vein occlusion. In a review of 100 cases of neovascular glaucoma, causative diagnoses of diabetes mellitus and central retinal vein occlusion comprised more than 50% of the cases.[62]

Neovascularization of the iris is also referred to as rubeosis iridis (diabetica), a term originated by Salus, who observed iris neovascularization in diabetic patients in 1928.[129] As recommended by Wand, the more accurate term is *neovascularization of the iris* (NVI), which we will use instead of rubeosis iridis in this review.[157] In NVI, new blood vessels form on the anterior surface of the iris and in the chamber angle recess. They grow initially within the iris stroma from preexisting iris capillary beds found near the iris root and at the pupillary margin. Then fine tufts of vessels form anteriorly on the iris near the pupil or in the angle. Unlike normal iris vessels, these newly formed vessels leak fluorescein. The vessels may remain stationary for long periods of time, up to years, without problems. However, they often enlarge and are accompanied by fibrous tissue, thereby occluding the angle and causing a secondary neovascular glaucoma that responds poorly to therapy.[59] These new vessels can range from being small thin-walled vessels to a firm fibrovascular layer.[5]

Neovascular glaucoma (NVG) is characterized by high intraocular pressure and NVI and trabecular meshwork.[116] In NVG, in addition to NVI and anterior chamber angle, new vessels can also be found on the retina, optic disk, and ciliary body.[92,159] Many eyes become either blind or so painful from the high intraocular pressure that they need to be enucleated. The stimulus for neovascularization is likely to be retinal hypoxia, and the vessels of the iris are believed to be the most sensitive within the eye to angiogenic stimuli.[59]

Iris neovascularization is associated with a variety of conditions, as shown in Table 3. Gartner and Henkind identified numerous causes of NVI, but the list is still likely to be incomplete.[44] Of the conditions listed in Table 3, vascular disorders are associated with ischemia, whereas surgery, trauma, and systemic diseases are associated with inflammation. However, the two frequently coexist in disease states.

In neoplastic disease, it is thought that the tumors produce angiogenic factors that spill onto the iris and other ocular tissue. Shields et al studied optic nerve invasion by retinoblastoma and found that NVI as well as an exophytic growth pattern of the tumor were significant clinical factors related to tumor spread.[131] If the angiogenic factors from the tumor can spread onto the iris, causing NVI, it is not surprising that spread elsewhere would lead to a higher risk of metastasis. The exophytic growth pattern is likely to be related to angiogenesis as well. Recent studies have shown that the vascular architecture of an ocular tumor is directly related to the chances of neoplastic spread.[40,123] These studies demonstrated that the presence of vascular networks, defined as at least three back-to-back closed vascular loops, indicates aggressive tumor behavior and provides the most significant association with death from metastatic ocular melanoma.

No studies have been done on the prevalence of NVI in the general population. However, the incidence of NVI in patient populations of the two most common causes of NVI, central retinal vein occlusion (CRVO) and diabetes, have been reported by various groups. Studies investigating the prevalence of NVI among enucleated eyes have also been done, as described below.

A. ENUCLEATED EYE STUDIES

In 1967, Schultze reviewed the histology of all eyes received in the pathology laboratory in 1 year to assess the relative frequency of NVI in enucleated eyes.[129] Of the 870 total cases that were reviewed, 12% (105) showed new blood vessel formation on the anterior surface of the iris. Iris neovasculariza-

tion was seen most commonly in cases of enucleation from patients who had glaucoma secondary to occlusion of the central retinal vein. Eighty percent of those cases showed NVI.[129] Anderson et al in 1971 similarly reviewed all enucleations at the University of Toronto Eye Pathology Department during a 5-year period, starting January 1, 1964.[5] Of the 460 total eyes in the study, 15.2% (70) were confirmed to have NVI, and glaucoma had occurred in 75% of the eyes.

Walton and Grant investigated the incidence of NVI in cases of retinoblastoma by reviewing histologically all eyes enucleated for retinoblastoma.[156] Eighty-eight eyes provided adequate sections for review. Of these, iris vascularization was noted in 44%.

These prevalence values for NVI are clearly not generalizable to the general population. To necessitate enucleation, an advanced ocular disease is likely to have been present. These patients, therefore, probably suffered from painful glaucoma or blindness, which increases the likelihood of the associated neovascular findings. However, the numbers are of interest in demonstrating the frequency of NVI in association with serious eye conditions requiring enucleation.

In summary, the prevalence of NVI in enucleated eyes for which neovascularization was likely causal for pain and blindness ranged from 12% to 15%.[5,129] Of an estimated 11,000 enucleations per year (estimate done for 1976) in the USA, we can calculate that 1,320 (12%) to 1,650 (15%) enucleations are done per year for eyes with NVI (Table 1).[10] As mentioned before, the NVI and its sequelae, such as NVG, most likely led directly to the need for these enucleations. In children with retinoblastoma, enucleated eyes showed NVI in 44% of the eyes in 1968.[156] To make an upper-range estimate of these cases, we will apply this 44% prevalence of NVI to all retinoblastoma patients. Retinoblastoma occurred in approximately 1 in 20,000 live births in the USA during 1974 to 1985.[142] We can calculate that of the 69.5 million single live births per year in the USA (from 1982–1988 estimates), approximately 3,500 newborns will have retinoblastoma.[18] Thus, if 44% of these infants develop NVI, this worst-case estimate is 1,540 new cases of NVI from retinoblastoma per year (Table 1).

TABLE 3

Diseases Associated With Iris Neovascularization

Vascular disorders
Central retinal vein occlusion*
Central retinal artery occlusion
Branch retinal vein occlusion
Carotid occlusive disease
Takayasu disease
Giant cell arteritis
Cartotid artery ligation
Carotid-cavernous fistula
Leber ciliary aneurysms
Retinopathy of prematurity
Sturge-Weber disease with choroidal hemangioma
Ocular disease
Neovascular glaucoma*
Uveitis
Endophthalmitis
Vogt-Koyanagi syndrome
Retinal detachment
Persistent hyperplastic vitreous
Coats disease
Eales disease
Pseudoexfoliation of the lens capsule
Sympathetic ophthalmia
Surgery and radiation therapy
Retinal detachment surgery
Vitrectomy
Laser coreoplasty
Cataract extraction
Radiation
Trauma
Systemic diseases
Diabetes mellitus*
Norrie's disease
Sickle cell disease
Neurofibromatosis
Lupus erythematosis
Marfan syndrome
Neoplastic diseases
Retinoblastoma*
Melanoma of the choroid
Melanoma of iris
Metastatic carcinoma
Reticulum cell sarcoma of ciliary body

*Most frequently associated with iris neovascularization.
Modified from Gartner and Henkind.[44]

B. CENTRAL RETINAL VEIN OCCLUSION

As mentioned earlier, CRVO is the leading cause of NVI. After a CRVO, NVI often develops, followed by NVG.[44] The overall incidence of NVI in all CRVO cases is 12% to 30%.[134] Sinclair and Gragoudas studied the records of 57 patients with CRVO, including the clinical examination, color fundus photographs and fluorescein angiograms; 21% were found to develop NVI.[134] Keenan et al studied 235 patients with CRVO who attended an ophthalmology clinic during a 7-year period and reported a 13.2% incidence of NVI during that period.[72] They further matched, by age, sex, and ethnicity, a group without NVI to those who had developed NVI to investigate the association of underlying medical conditions with increased risk of developing NVI in CRVO. Their results showed no significant difference in risk except that increasing age was associated with the development of NVI. The mean age of patients with NVI was 65 years.[72]

Central retinal vein occlusion is often categorized as ischemic or nonischemic. The significance of this

distinction is that NVI is correlated most significantly with the ischemic type of CRVO.[134] Two thirds of all CRVOs are categorized as nonischemic, and one-third are ischemic.[103,114] Two-thirds of patients with ischemic CRVO, in turn, will develop neovascular complications, such as NVG.[103] This is consistent with the finding that the incidence of NVG with all CRVOs is between 20% and 33%.[16,103,116,150] Yet, with the ischemic type of occlusion, NVG develops in 50% to 60% of cases.[57,60] The glaucoma usually develops 3 months after the CRVO occurs.[44]

The principal factor influencing the development of ocular neovascularization in retinal vein occlusion (RVO) seems to be the severity and extent of retinal ischemia.[58,134] This is illustrated by the report of NVI in 45% to 80% of ischemic eyes, whereas NVI was found in less than 5% of nonischemic eyes.[58,134] In addition, neovascularization of the optic disk and retina occurred in 24% of ischemic CRVO cases.[158] Magargal et al, moreover, reported that the ischemic form of RVO was more frequent in older patients, who as a whole have a higher incidence of systemic vessel disease compared with younger patients.[102] This systemic vessel disease may cause a certain degree of hypoxia of the retina even without a retinal vein occlusion event. Laatikainen confirmed that the chances of developing NVG in the ischemic form of occlusion increase with age.[93]

Further evidence supporting that the extent of retinal ischemia directly affects the amount of neovascularization comes from a study by Hayreh et al.[58] A prospective study was conducted of 721 eyes with RVO to determine the incidence of various types of ocular neovascularization and the factors influencing their development. Serious ocular neovascularization attributable to RVO was seen only in the three ischemic types of RVO. The incidence rates of neovascularization were as follows: 57.7% for ischemic retinopathy, 12.9% for ischemic-type hemiretinopathy, and 1.6% for major branch RVO.[58] It is not surprising that NVG, which is common after CRVO, develops only rarely after a branch RVO, which causes the least hypoxia of the various types of RVOs.[17]

Progression of the nonischemic CRVO to the ischemic type is also noted in approximately one-tenth of nonischemic CRVO cases.[114] Once converted, they assume the same risks of neovascularization associated with ischemic CRVO cases. Quinlan et al retrospectively studied 226 eyes diagnosed with CRVO.[114] At the initial visit, 36% were ischemic and 64% were nonischemic. Nine percent of the nonischemic cases, however, converted to ischemic cases between 1 and 36 months after the initial visit. No significant risk factor, ocular or systemic, was identified in the converted group as compared with the group remaining nonischemic. Once converted, they had the same poor visual prognosis and incidence of complications as the ischemic group.

In summary, the overall incidence of NVI after CRVO is roughly 20%, with a range of reports from 12% to 30%.[134] The principal factor influencing neovascularization is retinal ischemia, as suggested by the finding that NVI occurs mostly in the ischemic type of CRVO. Iris neovascularization occurs in 45% to 80% of patients with ischemic CRVO.[58,134] There is also the risk of nonischemic CRVO progressing to ischemic in 1 of 10 cases.[114] Once converted, they assume the same risks of neovascularization as the ischemic cases.

Although there is no formally reported census of patients with CRVO in the USA, it can be estimated from a 4-year incidence rate, reported in 1988 by David et al, of 0.72 per 1,000 in a general population older than 40 years and 106 million people in this same age group from 1995 USA population data.[27,148] From the above figures, the incidence of new CRVO cases in the USA is estimated to be 19,000 per year. With NVI occurring in roughly 20% of these patients, an estimated 3,800 cases of new NVI as a result of CRVO can be expected each year (Table 1).

C. DIABETES

Neovascularization of the iris in diabetes appears most often after the development of proliferative retinopathy. The onset of glaucoma after the appearance of NVI with diabetes is usually after a period of many years, as compared with only a few months with CRVO.[44] The incidence of NVI in diabetic patients ranges between 1% and 17%.[16,143] In patients with proliferative diabetic retinopathy, NVI has been reported at much higher incidence rates, ranging from 33% to 64%.[101,111,143] In patients with severe proliferative diabetic retinopathy, neovascular glaucoma occurs in 5% to 8% of cases.[133] In addition, for diabetic patients with NVG in one eye, NVG developed in the second eye in one-third of the patients.[111]

Further data are provided by the large-scale Diabetic Retinopathy Study.[38] In that study, a randomized, controlled multicenter clinical trial testing the benefits of photocoagulation in preserving vision in patients with proliferative diabetic retinopathy was conducted. Of a total of 1,256 patients with proliferative retinopathy examined for specific abnormalities, 2.3% to 2.5% had new vessels observed on the iris by eye examination and angiography. This value is much lower than those cited earlier, and this discrepancy may be related to differences in the various studies' definitions and methods of detecting neovascularization. Based on the Klein et al estimate that 700,000 Americans in 1980–1982 had proliferative diabetic retinopathy, the estimated incidence of NVI

in this group is 16,100 (2.3%) to 17,500 (2.5%) (Table 1).[84] This is a low estimate because the Diabetic Retinopathy Study prevalence data on diabetic NVI are much lower than those in other studies. Also, these low prevalence figures were applied to the estimate by Klein et al of only 700,000 Americans with proliferative diabetic retinopathy, based on 6 million known diabetic Americans in 1980–1982.[84] It is currently reported that there are more than 14 million Americans with diabetes.[79]

D. SUMMARY

Neovascularization of the iris is seen widely, but it is most significant as a complication of CRVO and diabetes. It is seen in 20% of CRVO cases, representing an estimated 3,800 cases of new NVI caused by CRVO per year. Iris neovascularization occurs in 45% to 80% of the ischemic type of CRVOs.[58,134] One-third of CRVO cases are ischemic and two-thirds are nonischemic with a conversion rate of 10% becoming ischemic.[103,114] The main influence on NVI appears to be the extent of retinal ischemia.[58] In patients with diabetes, the prevalence of NVI is reported in the Diabetic Retinopathy Study as 2.3% to 2.5% of patients with proliferative retinopathy, representing at least 16,100 to 17,500 Americans.[33]

III. Retina

Diseases affecting the retina comprise a majority of the causes of severe loss of vision in developed countries. Diabetes mellitus, the main contributor to this group of diseases, is the leading cause of new blindness in working-age Americans and accounts for at least 12% of the new cases of blindness each year in the USA, blinding more than 8,000 people annually.[10,77,79,146] Neovascularization of the retina is a critical part of the disease process in not only diabetes, but also such conditions as retinopathy of prematurity (ROP) and retinopathy associated with sickle cell disease. Age-related macular degeneration may also be associated with retinal neovascularization. However, because the primary neovascularization originates in the choroid in age-related macular degeneration, this topic will be presented in the next section.

Neovascularization of the retina involves the growth of new capillaries from the vessels that arise from the optic disk or inner retina. The origin is usually from the venules, but they also may arise from arterioles. Initially, the fragile new capillaries lie in the plane of the retina, but they may extend onto the vitreous face and rupture, resulting in vitreous hemorrhage, which leads to loss of vision. The greatest threats to vision are scarring, tractional detachment of the retina, and hemorrhage. New vessels may be asymptomatic until these complications develop.[3]

Prevalence reports on diabetic retinopathy and ROP will be reviewed in this section, as well as a small number of prevalence studies on neovascularization seen with sickle cell retinopathy. In addition, conditions and factors affecting neovascularization in diabetic retinopathy, such as pregnancy, puberty, and racial background, will be presented. A list of diseases involving retinal neovascularization modified from Henkind[59] is provided in Table 4.

A. DIABETIC RETINOPATHY

Diabetes mellitus is a chronic disease with long-term macrovascular and microvascular complications, including diabetic nephropathy, neuropathy, and retinopathy. Although more than 16 million Americans have diabetes mellitus, only approximately 50% of people with this disorder are aware of it.[79] Diabetic retinopathy is the most common cause of blindness among Americans between the ages of 20 and 74 years.[10]

Diabetic retinopathy is classified as *nonproliferative* and *proliferative. Nonproliferative diabetic retinopathy* is characterized by venous dilation and beading, retinal hemorrhage, microaneurysms, intraretinal lipid exudates, and soft exudates (microinfarcts of the retinal nerve fiber layer). These changes occur in the original retinal vessels that are present since birth. They result mostly from occlusions of retinal capillaries and the capillary dilation or shunt vessel formation that follows. Macular ischemia may also cause vision loss.

In contrast, *proliferative diabetic retinopathy* is characterized by abnormal new vessel and/or fibrous tis-

TABLE 4

Diseases Associated With Retinal Neovascularization

Diabetes mellitus*
Age-related macular degeneration*
Retinopathy of prematurity*
Central retinal vein occlusion*
Branch retinal vein occlusion*
Sickle cell disease*
Systemic lupus erythematosus
Eales disease
Multiple sclerosis
Distal large artery occlusion
Takayasu disease
Carotid artery obstruction
Coats disease
Tumors
Retinal detachment

*Most frequently associated with retinal neovascularization.

Modified from Henkind.[59]

sue proliferation on the surface of the retina, which may extend onto the vitreous face. These findings are more serious and ominous.[3] Retinal neovascularization most commonly occurs along the temporal vascular arcades and on the optic nerve head. Vitreous hemorrhage is a major component of visual loss, because these new vessels are fragile and prone to bleeding. The proliferating vessels are often accompanied by glial tissue that can contract and lead to traction on the retina.[3] In the most severe cases, the fibrovascular tissue leads to complete retinal detachment. Retinal neovascularization is known to be the major contributor to severe visual loss in diabetes, and treatment of retinal neovascularization in proliferative diabetic retinopathy can reduce the likelihood of severe visual loss from 50% to approximately 5% per year.[31]

Additionally, diabetic retinopathy is a source of great financial cost. Studies on cost savings associated with detection of eye disease predicted savings of 79,236 person-years of sight and more than $167 million to the federal budget if all patients with type 1 diabetes mellitus receive appropriate eye care.[66] The savings are 94,304 person-years of sight and more than $472 million if all patients with type 2 diabetes mellitus receive appropriate care.[67] In these studies, the calculated savings from improved eye care and prevention programs for type 2 diabetes came almost exclusively from the detection and treatment of macular edema. For the type 1 diabetes study, two-thirds of all savings would result from treatment of proliferative diabetic retinopathy.[66] Thus, although no studies address the total financial costs related specifically to retinal neovascularization and its sequelae in diabetes, we can estimate savings of at least $111.3 million (two-thirds of $167 million), which are currently being spent on proliferative diabetic retinopathy, if improved eye care and treatment programs are implemented. The recommended improvements include prompt eye care once a patient develops proliferative retinopathy with high-risk characteristics.[66]

The wealth of data on the epidemiology of diabetic retinopathy has been gathered with various modalities and populations. For this review, information is organized and presented in several categories. First, the incidence of proliferative retinopathy in a diabetic population is presented based on data primarily from the Wisconsin Epidemiological Study of Diabetic Retinopathy (WESDR) study.[85-87] Second, the prevalence of retinal neovascularization in the general population is presented based on data from the Framingham Eye Study and Klein population-based studies.[78,96] Third, the data on conditions affecting the prevalence of proliferative retinopathy, such as pregnancy, puberty, race, and other risk factors, are reviewed to potentially uncover factors significant to pathophysiology.

1. Retinopathy in the Diabetic Population

The largest study of retinal neovascularization within a diabetic population was the WESDR. In a population-based study in southern Wisconsin, Klein et al studied the prevalence and incidence of proliferative retinopathy in more than 2,000 patients with diabetes.[85-87] Data were reported in the following two groups: people less than 30 years old at time of diagnosis of diabetes and those 30 years old or older at the time of diagnosis. In the group that was less than 30 years old at diagnosis, 23% of 996 of the patients, all taking insulin, had proliferative retinopathy as a complication.[87] The prevalence of all retinopathy varied from 17% in patients with diabetes for less than 5 years to 97.5% for those with diabetes for more than 15 years. The prevalence of proliferative retinopathy increased with greater duration of diabetic disease as well and varied from 1.2% in patients with diabetes for less than 10 years to more than 67% in those with diabetes for more than 35 years.[87]

Similar data were reported for those 30 years old or more at diagnosis. Of 1,370 older-onset subjects, 10% of the insulin-taking and 3% of the non-insulin-taking diabetics had proliferative retinopathy.[86] Furthermore, the prevalence of all types of diabetic retinopathy varied from 28.8% in persons with diabetes for less than 5 years to 77.8% in those having had diabetes for 15 or more years. The prevalence of proliferative retinopathy similarly ranged from 2.0% to 15.5% in persons with diabetes for less than 5 years to those with diabetes for more than 15 years, respectively.[86] The study concluded that the frequency and severity of retinopathy is strongly associated with the duration of diabetes. Severity was also related to a younger age at diagnosis, higher glycosylated hemoglobin levels, higher systolic blood pressures, insulin use, proteinuria, and a small body mass.[86]

Based on these 1980–1982 data and 6 million known diabetic Americans, Klein et al estimated that 700,000 people in the USA had proliferative diabetic retinopathy, with 130,000 of those having retinal lesions associated with a high risk for severe vision loss (visual acuity less than 5/200 at two or more consecutively completed examinations at 4-month intervals) (Table 1).[84] These lesions are called Diabetic Retinopathy Study high-risk characteristics, and they were defined in the original study as "1) presence of vitreous or preretinal hemorrhage, 2) presence of new vessels, 3) location of new vessels on or near the optic disk, and 4) severity of new vessels."[32] It should be noted that this estimate of 700,000 people with proliferative diabetic retinopathy, based on 6 million

diabetic Americans, is a low estimate because it is currently reported that there are more than 14 million Americans with diabetes.[79]

These numbers may also underestimate the overall prevalence of neovascularization because they did not include very early vascular changes, called intraretinal microvascular abnormalities (IRMAs). These IRMAs, which describe irregular, segmental dilation of retinal capillaries, had previously been regarded as only a precursor to neovascularization. From current studies, such as cross-sectional morphology data on eyes with IRMAs, it is thought by some that IRMAs may be a form of neovascularization.[107,147] Intraretinal microvascular abnormalities are still currently defined as a form of nonproliferative retinopathy, and the debate regarding their designation as a form of neovascularization is ongoing within the ophthalmic community. However, because this study is interested in assessing all aspects of possible neovascularization, we will retabulate the prevalence of retinal neovascularization from the original Klein et al data to include IRMAs as a possible form of neovascularization.[86,87] Including IRMAs, the prevalence of neovascularization is retabulated to be 40% (versus 23%) of the younger-onset diabetics. For the older-onset diabetics, the new values are 30% (versus 10%) for the insulin-taking patients and 10% (versus 3%) for the non–insulin-taking patients. Because our new values are roughly three times the previous values in the older-onset group, which represents most of the cases of diabetes, we will multiply the previous 1980–1982 estimate by Klein et al of 700,000 people with proliferative diabetic retinopathy by three. Thus, we estimate that 2.1 million people in the USA have all types of retinal neovascularization, including IRMAs, related to diabetes (Table 1). As described above, this may also be a low estimate because there are currently more than 14 million diabetic Americans, and the Klein et al estimate was based on 6 million diabetic Americans.[79]

To complete the picture of the magnitude of proliferative diabetic retinopathy, it is important to anticipate the progression of diabetic retinopathy over time. Klein et al followed up with the WESDR group of patients in a population-based study examining the 10-year incidence and progression of diabetic retinopathy.[85] The overall 10-year incidence of any retinopathy was 74%. Progression of all forms of retinopathy occurred in 64% of cases during the 10 years, and progression to proliferative retinopathy occurred in 17% of cases overall. The breakdown for 10-year incidence of progression to proliferative retinopathy was as follows: 30% in younger-onset diabetics, 24% in insulin-taking older-onset diabetics, and 10% in non–insulin-taking older-onset diabetics. These high incidence rates of progression occurred despite improvements in the treatment of insulin-dependent diabetes mellitus during this time period. Furthermore, the findings showed an increase in the annual incidence of proliferative retinopathy within the older-onset group during this 10-year span. This increase in annual incidence is significant for anticipating and planning for necessary services.[85] This 1980–1992 study estimated that during a 10-year period, 915,000 (15.8%) of 6 million Americans with known diabetes will develop proliferative retinopathy, of which 320,000 (5.5% of diabetic Americans) will develop lesions associated with a high risk of severe vision loss.[85] Therefore, this study suggests an estimated incidence of 91,500 new cases of proliferative retinopathy per year (Table 1). This is a low estimate because it is currently reported that there are more than 14 million Americans with diabetes.[79]

Other reports on proliferative retinopathy in a diabetic population include studies in small populations, such as the Pittsburgh Epidemiology of Diabetes Complications Study.[91] Of 657 insulin-taking diabetic patients analyzed by stereoscopic fundus photographs, 31% had proliferative retinopathy, and 53%, nonproliferative retinopathy. These values are higher than the WESDR prevalence findings, and the differences are likely caused by population selection. The Pittsburgh study examined only insulin-taking subjects, whereas the WESDR also included non–insulin-taking subjects, who tend to have fewer complications and less proliferative retinopathy. In another study, by the Chinese Academy of Medical Sciences, 662 cases of diabetes mellitus were examined by mainly ophthalmoscopy, and 50 cases were studied with fluorescein angiography.[164] Prevalence of all diabetic retinopathy was 51.3%, with proliferative retinopathy found in 7.6% of patients. These values are lower than those in the earlier two studies. The grading scheme was not described, and this difference could possibly account for the discrepancy between the prevalence rates.[164] Because the estimate of the total number of cases of proliferative retinopathy and the 10-year incidence estimates from the WESDR are based on a total of 6 million known diabetics, we will calculate the prevalence estimates from these other studies (7.6% and 31%) using 6 million people with known diabetes for ease of comparison among the studies. From the Chinese Academy and Pittsburgh prevalence values, we can estimate 456,000 (7.6%) to 1.9 million (31%) people with proliferative diabetic retinopathy in the USA.[91,164] Yet, because it is now estimated that there are 14 million diabetics in the USA, many of whom are unaware of their condition, these same prevalence values (7.6% to 31.0%) may even represent 1.1 million to 4.3 million diabetics with proliferative disease. However, given the large size of the WESDR

population and systematic grading system for assessing retinal lesions in their study, the estimates based on WESDR data are likely to be more reliable.

To summarize the main points, the WESDR reported that 23% of younger-onset diabetics, 10% of insulin-taking older-onset diabetics, and 3% of non–insulin-taking older-onset diabetics had proliferative retinopathy.[86,87] Their estimate for the total number of people with proliferative diabetic retinopathy in the USA is 700,000.[84] From our retabulation of their original prevalence data, we estimate that 2.1 million people in the USA have all types of retinal neovascularization, including IRMAs, related to diabetes. Based on the WESDR incidence findings, it is estimated that during a 10-year period, 915,000 (15.8%) of 6 million Americans with known diabetes will develop proliferative retinopathy, of which 320,000 (5.5% of diabetic Americans) will develop lesions associated with a high risk of severe vision loss.[85] Thus, this study indicates an estimated incidence of 91,500 new cases of proliferative retinopathy per year. Other reports on the prevalence of proliferative retinopathy in diabetics span from 7.6% to 31.0% and may be less reliable than the WESDR data.[91,164]

2. Retinopathy in a General Population

The prevalence of retinopathy in diabetics is important in illustrating its significance as a disease complication. Yet, the prevalence of retinal neovascularization within a general population as opposed to only a diabetic population is useful in illustrating its overall impact. Such data could be extracted from the abundant data recorded in the population-based Framingham Eye Study.[96] The Framingham Eye Study was a large-scale prospective study in which ophthalmologic examinations were done on 2,631 persons (aged 52–85 years). Of this total group, the prevalence of diabetic retinopathy was 2.5% (67) of these patients, or 2.0% (107) of the 5,262 eyes examined. Also, of 156 eyes suspected of having diabetic retinopathy referred for a definitive examination, 6.4% (10 eyes) showed neovascularization of the disk or retina.[96] The number of patients representing these suspected diabetic retinopathy eyes (156) and eyes with neovascularization (10) were not reported. Because the 156 eyes analyzed represent as few as 78 people or as many as 156 people, it is difficult to accurately estimate the frequency of retinal neovascularization in a small group of people. Among the suspected diabetic patients, the prevalence of neovascularization could range from the smallest possible value of 3.2% (five people with neovascularization of 156 total) to the largest possible value of 12.8% (10 people with neovascularization of 78 total). Thus, these data clearly cannot be used to accurately estimate the prevalence of neovascularization in diabetic patients.

We can, however, estimate more accurately the prevalence of neovascularization in the general population from their data. Because 10 eyes represent as few as 5 people or up to 10 people, we will calculate the prevalence of retinal neovascularization in the general population using the median value of 7.5. Thus, we estimate a prevalence of retinal neovascularization of 0.3% (7.5 people with neovascularization of 2,631 total) of people in the general population (aged 52–85 years). Although the number of patients represented by 10 eyes was not known, this is a relatively accurate calculation of the prevalence from their data because of the large denominator (2,631). It should be noted that their estimated number of cases of neovascularization is likely to be much lower than the actual number because their study methods included using ophthalmoscopy alone as a screening method, without the use of stereoscopic fundus photographs of all study participants. Small neovascular changes, including IRMAs, could be easily missed by their screening protocol. Despite these caveats, we can apply their prevalence of 0.3% to the 60 million Americans in the same age range (52–85 years [from 1995 USA population data]) to make a low estimate of at least 180,000 people in the USA having proliferative diabetic retinopathy.[148]

Additional estimates of the prevalence of retinal neovascularization in the general population can be extrapolated from a population-based study by Klein.[78] This study reported the prevalences of various retinal lesions associated with diabetic retinopathy in a general population consisting of those with diabetes mellitus and those without diabetes or known retinal vascular disease. It included 4,797 people (aged 43–84 years) and used stereoscopic fundus photographs of all participants. Unlike the Framingham Eye Study and the majority of other studies, this study took into account all findings of retinal neovascularization, including the earliest lesions, IRMAs.

Of 4,311 subjects in whom diabetes was not known to be present, 0.5% (23) had IRMAs, which were the only type of neovascular changes seen in this group of people.[78] Of 427 subjects with known diabetes mellitus, 13.0% (57) showed evidence of all forms of retinal neovascularization. When combined, the total prevalence of retinal neovascularization in the entire study population (aged 43–84 years) of those with and without diabetes was 1.7% (80/4,738). Not surprisingly, Klein's value is much higher than the Framingham value (1.7% versus 0.3%), owing to the more sensitive study methods (fundus photographs) and inclusion of cases with IRMAs. Extrapolating this 1.7% prevalence to the 90.2 million Americans in 1995 in the same age range (43–84 years), we can

estimate that 1.5 million Americans in the indicated age range have diabetes-related retinal neovascularization, including IRMAs (Table 1).[148]

In this same study, Klein also reports a positive predictive value of various retinal lesions for the presence of diabetes mellitus.[78] The presence of blot hemorrhages has a predictive value of only 13.2%, and microaneurysms, 16.6%. Yet, more severe retinopathy has a predictive value of 62.3%, and proliferative retinopathy is 100% predictive of diabetes mellitus. Overall, this study demonstrates that retinal neovascularization is surprisingly prevalent, particularly in an undiagnosed diabetic population.[78] No population-based studies have been done on the incidence of retinal neovascularization secondary to diabetes in a general population.

As a summary, a low estimate of the prevalence of proliferative diabetic retinopathy in a general population was reported at 0.3% in the Framingham Eye Study, representing at least 180,000 people (aged 52–85 years) in the USA with neovascularization.[96] A retabulation of the data from Klein et al found the overall prevalence of all diabetic retinal neovascularization, including IRMAs, to be 1.7% of the general population, representing 1.5 million Americans aged 43–84 years.[78]

3. Conditions Affecting Diabetic Retinopathy

a. Pregnancy

In diabetic women, retinopathy is encountered in 25% of pregnancies.[120] Studies show a retinopathy incidence of 13.0% to 40.5% in pregnant diabetic patients.[94] When retinopathy is present at baseline, pregnancy is believed to contribute to its progression. A number of studies have been done to assess the progression of retinopathy in diabetic women during pregnancy, and the results have been variable.

Rodman et al tabulated the reported cases of retinopathy in pregnancy from previous studies.[120] A total of 201 cases of pregnant diabetic women with nonproliferative retinopathy was compiled. Of these, 10% showed progression of their disease during the 9 months of pregnancy. The authors concluded that this percentage is similar to that anticipated from a nonpregnant group, as retinopathy is known to progress on its own. Of 127 subjects with proliferative disease, however, 25% had progression of their retinopathy during pregnancy.[120] This finding suggested that pregnancy might pose additional risks to patients with proliferative retinopathy. Laatikainen et al similarly showed that in a prospective study of 73 pregnant diabetic women, those with minimal or no retinopathy at baseline had no significant progression.[94] However, 65% (13) of those with frank retinopathy during the first trimester showed progression during pregnancy.[94] Rosenn et al studied prospectively 154 women with insulin-dependent diabetes mellitus during pregnancy, of whom 33% had progression of their retinopathy during pregnancy. They found that progression of retinopathy during pregnancy was associated with the severity of underlying retinopathy, duration of diabetes, and chronic hypertension.[121]

Klein et al addressed the questions posed by the above authors in a prospective study to determine whether pregnancy poses an additional risk of worsening retinopathy in diabetic women.[76] A group of pregnant diabetic women were matched to nonpregnant diabetic women. Of 110 pregnant women without proliferative diabetic retinopathy initially, 7.3% progressed to a severe proliferative retinopathy state. Of 190 matched nonpregnant diabetic women without proliferative retinopathy, 3.7% progressed to severe proliferative retinopathy by the end of the same amount of time. Their findings showed that pregnancy was significantly associated with progression, with an adjusted odds ratio of 2.3.

b. Puberty

Data suggest that being prepubertal has a protective effect for the development of retinopathy in diabetics. In the WESDR data, proliferative retinopathy was not present in any participant less than 16 years old.[84] Those who were postmenarchal in the WESDR were 3.2 times as likely to have diabetic retinopathy as those who were premenarchal, after controlling for other factors. A review by Kimmel et al showed that proliferative retinopathy in preadolescents is exceedingly rare.[74] Fluorescein angiograms of 4,547 diabetic patients with clinically suspected retinopathy were studied. When a 13-year-old, prepubescent boy was found to have proliferative disease, a review of the available literature by the authors confirmed that his was the youngest documented case of proliferative diabetic retinopathy, and nearly no other prepubescent cases had been reported.[74]

c. Race

There are few population-based studies that compare the prevalence of proliferative retinopathy among many different racial groups. Studies within individual racial groups, however, have shown certain populations with high prevalence rates of proliferative retinopathy, such as Pima Indians and Mexican-Americans. Pima Indians, who have the highest prevalence and incidence of non–insulin-dependent diabetes mellitus (NIDDM), have been reported to have a 20-year cumulative incidence of proliferative diabetic retinopathy of 14%.[108] Similarly, Mexican-Americans also have a high prevalence of NIDDM and diabetic

retinopathy, particularly the preproliferative and proliferative forms.[56] Compared with non-Hispanic whites with NIDDM, Mexican-Americans with known NIDDM had a higher frequency of proliferative retinopathy (7% versus 3%). Future epidemiologic and genetic studies are needed to understand these racial differences and to uncover the basis for the high frequencies of proliferative diabetic retinopathy.

4. Other Risk Factors

Various studies have identified risk factors associated with the development and progression of diabetic retinopathy. Rand et al identified four factors that affect the risk of proliferative diabetic retinopathy.[115] These included frequency of hyperglycemia (defined as blood glucose levels above 200 mg/dL), less effort in managing diabetes as manifested by less frequent testing of urine for glucose, presence of certain human leukocyte antigen (HLA)–DR phenotypes, and less refractive error. The authors stated that the difference in risk based on refractive error did not seem significant at first, but it was significant when assessed among patients with one of the HLA-DR subtypes associated with increased susceptibility to proliferative retinopathy.[115] It has been previously noted that myopic eyes may be less damaged by diabetes than nonmyopic eyes. One theory posed by Rand et al is that there is decreased retinal blood flow in myopic eyes, which may confer a protective effect that mimics photocoagulation.[115]

Klein et al also examined risk factors for proliferative diabetic retinopathy.[84] They reviewed factors such as race, age, genetics, duration of diabetes, glycemia, blood pressure, cigarette smoking, alcohol, physical activity, proteinuria, oral contraceptives, pregnancy, and ocular factors, including myopia and glaucoma. Of these, a significantly increased risk of developing proliferative retinopathy was associated with hyperglycemia, longer duration of diabetes, and more severe retinopathy at baseline. High blood pressure at baseline was associated with an increased risk of proliferative diabetic retinopathy only in the younger-onset group, and progression to proliferative retinopathy was seen in pregnant diabetic women.[84]

To investigate other factors related to proliferative retinopathy, a recent study described the interval between first appearance of mild nonproliferative diabetic retinopathy and the first appearance of neovascularization in type 1 diabetes.[155] The authors concluded that later onset of mild nonproliferative retinopathy was not necessarily associated with delayed development of neovascularization in type 1 diabetes. This finding will likely affect intervention strategies, as delaying the onset of nonproliferative disease was not shown to forestall the development of neovascularization and its damaging effects.

5. Summary

Studies of the prevalence of proliferative retinopathy in diabetic populations demonstrate the frequency of retinal neovascularization as an ominous complication of diabetes. The WESDR reports proliferative retinopathy in 23% of diabetics diagnosed when less than 30 years old; in older-onset diabetics it is found in 10% of insulin-taking and 3% of non–insulin-taking subjects.[86,87] The duration of diabetes is correlated with the severity of retinopathy. Furthermore, their estimate for the total number of people with proliferative diabetic retinopathy in the United States is 700,000.[84] From our retabulation, we estimate that 2.1 million people in the USA have all types of retinal neovascularization, including IRMAs, related to diabetes. Based on the WESDR incidence findings, it is estimated that during a 10-year period, 915,000 (15.8%) of 6 million Americans with known diabetes will develop proliferative retinopathy, of whom 320,000 (5.5% of diabetic Americans) will develop lesions associated with a high risk of severe vision loss.[85] Other reports on the prevalence of proliferative retinopathy in diabetics span from 7.6% to 31.0% and are probably less reliable than the WESDR data.[91,164]

The overall magnitude of proliferative retinopathy is better estimated by prevalence studies in a general population. A low estimate of the prevalence of proliferative diabetic retinopathy in a general population was reported at 0.3% in the Framingham Eye Study, representing at least 180,000 people (aged 52–85 years) in the USA with neovascularization.[96] Data from Klein found the overall prevalence of all diabetic retinal neovascularization, including IRMAs, to be 1.7% of the general population, representing 1.5 million Americans aged 43–84 years.[78]

The prevalence data with certain physiologic conditions, such as pregnancy and puberty, suggest their influential role in the progression of diabetic retinopathy. Although reports of the progression rates of retinopathy in pregnant diabetic women are variable, pregnancy has been shown to be significantly associated with progression, with an adjusted odds ratio of 2.3.[76] The virtual absence of reported cases of proliferative retinopathy in prepubescent patients suggests that risks for developing retinopathy are incurred only after puberty occurs.[74] Racial factors are also important, as Pima Indians and Mexican-Americans have been reported to have high frequencies of proliferative retinopathy.[56,108] Finally, there is consistent evidence of an increased risk of proliferative retinopathy associated with longer duration of diabetes, hyperglycemia with poor control,

and more severe retinopathy at baseline.[84,115] The implications of these findings for prevention will be discussed in a later section.

B. RETINOPATHY OF PREMATURITY

Retinopathy of prematurity (ROP), a disease affecting premature infants, is characterized by retinal neovascularization that eventually includes the vitreous and often leads to retinal detachment and finally blindness. Two major phases of ROP are described. The acute phase either resolves or leads to the permanent sequelae of the cicatricial phase.[110] According to the International Classification of Retinopathy of Prematurity, ROP can be divided into five stages, with retinal neovascularization and its devastating sequelae being present in stages 3, 4, and 5.[23,65] In stage 1, a distinct demarcation line separating the not-yet-vascularized anterior part of the retina from the vascularized posterior part can be seen. In stage 2, this line has become a prominent ridge, and some tufts of newly formed vessels may be seen. Stage 3 is characterized by extraretinal fibroneovascular proliferation, which may extend from the retina onto the vitreous. In stage 4, the fibroneovascular proliferations have led to a partial-traction retinal detachment. Finally, in stage 5, formerly called retrolental fibroplasia, there is a characteristic funnel-shaped total retinal detachment.

It was discovered in the 1940s that high concentrations of oxygen in newborns' incubators contributed to the development of ROP. Incidence declined as oxygen was restricted, but despite this adjustment, the incidence of ROP has seen a resurgence because of the ability to save lower-birth-weight infants. Data collected by the Swiss Neonatology Group showed that the incidence in 1983–1985 of all stages of ROP in surviving infants with a birth weight less than 1,000 g was 16%, and 2% had neovascular ROP changes.[11] In 1989–1991, for this same birth weight category, the incidence of all stages of ROP had doubled to 33%, and 6% had neovascularization.

Recent studies report the incidence of ROP as varying from 10% to 30% in infants born weighing less than 1,500 g.[6] Archambault and Gomolin carried out a retrospective study on the incidence of ROP among 157 neonates weighing 2,000 g or less in the neonatal intensive care unit. Overall, they detected ROP in 15% of the cases. Of these, 75% weighed less than 1,000 g at birth. Their finding that low birth weight is a major risk factor for retinopathy confirms the results of earlier studies.[6] Similarly, Valentine et al found an incidence of ROP of 13% in 806 neonates in a neonatal intensive care unit born weighing less than 1,750 g.[149] This study went further to investigate the resurgence of ROP and its associations. They showed that the increase in the number of infants with ROP during the study period was caused by an increased survival rate of neonates rather than by iatrogenic measures as had occurred in the 1940s. Again, incidence rates of ROP were higher in lower-birth-weight infants.[149]

Epidemiologic data from community studies differ considerably from the above neonatal intensive care unit data. Ng et al found the incidence of acute ROP to be 49.1% in 505 infants born weighing less than 1,700 g whom were followed up in a community practice.[110] Most cases resolved, and the cicatricial sequelae leading to blindness occurred in only 2.0% of the low–birth-weight infants. The higher overall incidence of ROP is likely related to the community-based nature of the study. More frequent and longer follow-up examinations probably allowed better detection of ROP of all stages. In their study, most of the cases of ROP resolved, but it is a serious disease because of the blinding effects in some of the cases.[110]

The incidence and severity of ROP are inversely related to the gestational age of the premature newborn.[11] Thus, retinal neovascularization and its sequelae can be seen most frequently in the youngest premature newborns. A synthesis of the results of many studies shows that in the youngest category of premature newborns (born at 24–27 weeks of gestation), a neovascular stage of ROP occurred in 12% to 29% of the newborns; in the middle category(28–31 weeks of gestation), neovascular changes occurred in 2% to 20%; and in the oldest group (32–35 weeks of gestation), neovascularization was present in only 0% to 3.5% of infants.[12,70,73,110,112,113,117,125,162,163]

Despite meticulous neonatal care, ROP in advanced stages still occurs. Incidence reports of all stages of ROP vary from 10% to 30% of low-birth-weight infants, with several studies consistently reporting values of 13% to 15%.[6,149] Based on the National Center for Health Statistics report, 5.1 million low–birth-weight (defined as less than 2,500 g) infants were born in the USA in 1988.[18] Breakdown data on smaller-weight newborns were not reported. Because most of the 5.1 million newborns (less than 2,500 g) probably represent older premature infants, we can estimate the number of newborns with neovascular ROP by applying the above prevalence findings of 0% to 3.5% to this number.[12,70,73,110,112,113,117,125,162,163] Thus, an upper estimate of the incidence of retinal neovascularization secondary to ROP is 180,000 new cases per year (3.5% of 5.1 million per year [Table 1]). A community-based study with frequent follow-up found that most cases of ROP resolve and that only 2.0% develop blinding cicatricial sequelae.[110] The present resurgence of ROP is caused by the higher risk of ROP in lower–birth-

weight infants, numbers of whom continue to increase as survival rates increase.

C. SICKLE CELL RETINOPATHY

In patients with sickle cell disease (hemoglobin SC disease) and other sickling disorders, there is a high prevalence of retinal neovascularization and its sequelae, as well as occlusion of small blood vessels in the eye. Occlusion of larger vessels, such as a central retinal artery occlusion, may also occur, but at a lower frequency.[20] Some of the ocular lesions in sickle cell patients, such as the "comma sign" (comma-shaped blood vessels) in the conjunctiva and angioid streaks in the fundus, are common but do not need to be treated. Angioid streaks are seen in about 1% to 2% of a sickle cell clinic population, and their prevalence increases in older patients, being greater than 20% in SS patients older than 40 years.[24] Angioid streaks usually are not pathologic, but they must be followed up closely because they can occasionally lead to neovascularization that can bleed into the macula and thereby decrease vision.

Like diabetic retinopathy, sickle retinopathy is divided into a nonproliferative and proliferative form, and accordingly the nonproliferative form is less serious. Proliferative sickle retinopathy is the most severe ocular complication of sickle cell disease and sickling disorders. In proliferative sickle retinopathy, the occlusion of small blood vessels in the peripheral retina may lead to the enlargement of existing capillaries and retinal neovascularization. Tufts of neovascular tissue, called "sea fans" because of their shape, can grow onto the vitreous and along the surface of the retina.[51] Thus, proliferative sickle retinopathy is characterized by peripheral arteriolar occlusions, arteriolar-venular anastomoses, neovascular proliferation, vitreous hemorrhage, and retinal detachment.[50]

Extensive population-based studies on the prevalence and incidence of proliferative sickle retinopathy have not been done. A few studies have examined the presence of sickle cell retinopathy within small population groups. Talbot et al observed retinal changes, not including proliferative retinopathy, in more than 30% of children with sickle cell anemia as young as 5.0 to 7.5 years of age.[141] These changes are less prevalent in children with hemoglobin SC disease, which is in striking contrast to the higher prevalence of proliferative retinopathy in adults with SC disease. In fact, the proliferative or neovascular form of sickle cell retinopathy is more common in adults with hemoglobin SC disease (in 40%) than it is in adults with sickle cell anemia (in 20%), and the prevalence increases with age for both.[25,42]

In the USA, most of the cases of sickle cell anemia and hemoglobin SC disease are seen in the African-American population. Because the prevalences of sickle cell anemia (0.14%) and hemoglobin SC disease (0.13%) among African-Americans were reported in 1981, we can calculate an estimate of the actual number of people with neovascularization secondary to sickle cell retinopathy in the USA.[160] According to USA 1995 population data, there are 19.7 million African-Americans (aged 18–64 years).[148] Thus, approximately 27,580 (0.14%) adult African-Americans have sickle cell anemia, and 25,610 (0.13%) have SC disease. We therefore can estimate that there are 5,500 (20% of 27,580) cases of proliferative sickle retinopathy in African-Americans (aged 18–64 years) with sickle cell anemia (Table 1). For adult African-Americans with hemoglobin SC disease, the number is higher, an estimated 10,200 (40% of 25,610) people with neovascular sickle retinopathy (Table 1). Much work remains to be done to explain the epidemiologic patterns seen with sickle cell retinopathy.

IV. Choroid

Choroidal neovascularization (CNV) is defined as the formation of new blood vessels that are located between the retinal pigment epithelium (RPE) and Bruch membrane and are continuous with the normal choroidal vessels.[52] Vision loss related to CNV is significant because of its generally poor prognosis, although in some eyes CNV may be amenable to laser treatment.[124]

Clinically, ophthalmoscopy and fluorescein angiography can be used to detect CNV. Ophthalmic signs of CNV include a subretinal membrane, hemorrhagic detachment of the retina and/or RPE, intraretinal or subretinal exudates in the absence of retinal vascular disease, sub-RPE ring lesions, and serous detachment of the RPE, particularly when associated with an indentation or notch of the margin of the RPE detachment or radial chorioretinal folds surrounding the RPE detachment.[46,47]

Choroidal neovascularization may occur in a variety of different conditions, as listed in Table 5.[53] Although the greatest number of studies focus on age-related macular degeneration, a disease of the elderly that is presented in the next subsection, much less attention has been placed on CNV in patients younger than 50 years. Cohen et al reviewed the literature on these younger patients and determined the relative frequency of the various causes of CNV.[21] Of 363 patients less than 50 years old who had CNV, Cohen et al found that high myopia was seen most frequently, in 62% (225) of the cases. The remainder of the cases were associated with the following conditions: presumed ocular histoplasmosis syndrome in 42 (12%), angioid streaks in 17 (5%), and miscel-

laneous hereditary, traumatic, or inflammatory disorders in 16 (4%). In 63 of the patients (17%), CNV was not found to be related to any cause and was labeled idiopathic. Of the patients with myopia and CNV, the neovascularization was subfoveal in 62%, compared with much lower prevalence rates of 30% to 36% in patients with other presumed causes.

A. AGE-RELATED MACULAR DEGENERATION

Age-related maculopathy (ARM), also referred to as age-related macular degeneration (abbreviated AMD or ARMD), is the leading cause of severe, irreversible visual loss in elderly Americans.[96,146] Funduscopic findings include one or more of the following: the presence of drusen (yellow deposits below the RPE); hyperpigmentary and hypopigmentary changes of the RPE; atrophic macular degeneration (well-defined areas of atrophy, called geographic atrophy, or other atrophy of the RPE and choriocapillaris); and neovascular macular degeneration (CNV, serous or hemorrhagic detachment of the RPE, and subsequent scarring of the macular area).[154] To facilitate the comparison of data from different epidemiologic studies, an international classification system has defined AMD as all manifestations of this disorder and AMD as the late-stage form, characterized by either the neovascular/exudative or nonneovascular atrophic macular degeneration.[9]

The development of CNV has devastating effects on the visual capabilities of the patient and is the major contributor to loss of vision in AMD.[15] Severe visual loss occurs almost exclusively in eyes with neovascular AMD as compared with those with nonneovascular changes.[15] The eyes of AMD patients without CNV have an average visual acuity loss over 5 years of only 0.4 lines.[15] However, in patients with extrafoveal CNV in both eyes, 49% became blind from this neovascular form of AMD within 5 years.[99]

Several groups have studied the prevalence of this disease, which is useful for planning ophthalmologic care strategies. Comparing the prevalence rates from different populations can also provide clues regarding the etiology of AMD.[154] Cross-sectional population studies of AMD have been done in the USA, Denmark, Iceland, Netherlands, and New Zealand.[13,48,68,75,82,96,106,152,153] All of these groups demonstrate that the prevalence of ARM increases dramatically with advancing age. In a compilation of these studies, the overall prevalence for any type of AMD is approximately 20% in the 65- to 74-year-old age group and 35% in the 75- to 84-year-old population.[154] Applying these figures to 1995 USA population data, an estimated 3,751,800 Americans aged 65–74 years and 3,900,750 Americans aged 75–84 years have some form of AMD.[148] Data on the prevalence of the neovascular form of macular degeneration, which is chiefly responsible for vision loss, are available in the Beaver Dam, Chesapeake Bay, Framingham, Iceland, and Rotterdam studies (Table 6).[13,68,82,96,153]

In the Beaver Dam Eye Study, Klein et al examined the prevalence of AMD in a general population (4,926 participants) between the ages of 43 and 86 years in Beaver Dam, Wisconsin.[82] The overall prevalence of the late forms of AMD in the Beaver Dam

TABLE 5

Diseases Associated With Choroidal Neovascularization

Degenerative conditions
Age-related macular degeneration*
Angioid streaks*
Nodular and diffuse drusen
Macular area
Peripapillary area
Peripheral area
Myopia
Fuchs dot
Lacquer cracks
In the macula in pathologic myopia
Osteogenesis imperfecta
Drusen of the optic nerve head
Optic nerve head pits
Retinochoroidal coloboma
Best's disease
Retinitis pigmentosa with marked exudation
Inflammatory or infectious conditions
Ocular histoplasmosis syndrome*
Sarcoidosis*
Toxoplasma retinochoroiditis
Rubella
Vogt-Koyanagi-Harada syndrome
Birdshot retinochoroidopathy
Behçet's disease
Chronic uveitis
Tumors
Choroidal nevi
Malignant melanoma
Choroidal hemangioma
Extrapapillary hamartomas of the retinal pigment epithelium
Choroidal osteomas
Trauma
Choroidal rupture
Photocoagulation (xenon or argon)
Complicating drainage of subretinal fluid
Retinal cryoinjury
Miscellaneous
Serpiginous or geographic choroiditis
Idiopathic in macular area with central serous retinopathy–like picture
Idiopathic in macula
Fundus flavimaculatus
Punctate inner choroidopathy
Chronic retinal detachment
Neovascularization from pars plana and/or choroid at ora serrata

*Most frequently associated with choroidal neovascularization.

Modified from Green and Wilson.[53]

study was reported at 1.6% of the studied population (aged 43–86 years). The exudative form of AMD, characterized by CNV and its sequelae, was present in 1.2% of this same general population. Thus, the neovascular form composed 75% (1.2%/1.6%) of the AMD cases, and the nonneovascular atrophic form, roughly 25%. Applying the 1.2% prevalence value of neovascular AMD in a general population of 43–86-year-olds to 1995 USA population data, there are an estimated 1.1 million people in the USA with CNV secondary to AMD (Table 6).[148]

Prevalence data on neovascular AMD for smaller subgroups of the population divided by age (where available) are also shown in Table 6, as they can provide a more accurate estimate of the number of cases of neovascular AMD, because the population sizes of subgroups at different ages vary significantly. When prevalence data were reported for combined neovascular and atrophic AMD, the value was multiplied by 75% to estimate the prevalence of only neovascular AMD.[82] As an example, the National Society to Prevent Blindness used data from the Beaver Dam Eye Study and the 1990 United States Bureau of the Census population data to estimate that there are 13,163,865 cases of all types of AMD and 1,226,134 cases of late AMD, defined as the presence of geographic atrophy or exudative disease, among whites over 40 years old.[146] When the Klein et al prevalence findings are used to estimate that 75% of these cases of late AMD represent exudative disease, then roughly 920,000 white Americans over 40 years old have neovascular AMD (Table 6).

Extensive data on AMD among African-Americans and other nonwhite populations are not currently available, but all indications are that the prevalence of AMD, particularly the exudative form, is significantly lower than for whites.[55,126] A population-based study in East Baltimore, MD, reported that bilateral blindness caused by AMD was significantly more common among whites than African-Americans (16 of 2,913 whites compared to none of 2,395 African-Americans examined were blind as a result of AMD).[135] Klein et al also showed that AMD was more prevalent in whites (compared with nonwhites) over 60 years old.[89] Further studies are necessary to understand the cause of these epidemiologic differences.

The general prevalence of CNV was also reported in an occupational cohort of 777 men (aged 30–95 years) from the Chesapeake Bay region in Maryland, half of whom were under the age of 50 years.[13] The prevalence of neovascular AMD was found to be 0.5% in their overall population (aged 30–95 years), which represents an estimated 750,000 Americans in the same age group (Table 6).[13,148] More accurate estimates can be made with their breakdown data, such as a 6% prevalence of all forms (exudative and atrophic) of AMD in individuals 70 to 95 years old. Applying 75% to this value based on the Beaver Dam Eye Study results, we can calculate that 4.5% of this age group has neovascular AMD, or roughly 1.1 million Americans between the ages of 70 and 95 years.[148] The calculated estimated cases of neovascular AMD in the USA, based on the prevalence values from five studies, are compared in Table 6.[68,96,153] Notably, the lowest (Rotterdam, The Netherlands study) and highest (Icelandic study) estimates come from countries other than the USA and their populations may have a different prevalence of neovascular AMD and other factors (e.g., environmental, genetic, etc.), that complicate the extrapolation of prevalence data to the population of the USA.

To date, only two population-based studies of the incidence of AMD have been performed.[14,81] These studies include the incidence of neovascular AMD, which is immensely helpful for planning treatment strategies as well as estimating need for future services and associated costs related to severe vision loss. Klein et al provide data on the incidence and progression of AMD from a large, general population (3,583 participants, aged 43–86 years at baseline) in Beaver Dam, Wisconsin, in which the prevalence of AMD was studied earlier.[81,82] They found that the 5-year incidence of neovascular AMD was 0.6% of their total population (aged 43–86 years). Based on 1995 USA population data, we estimate a 5-year incidence of 550,000 new cases, or roughly 110,000 new cases per year of neovascular AMD for this age range (Table 6).[148]

In the Chesapeake Bay region, Bressler et al[5] estimated the 5-year incidence of AMD-related CNV as 0.2% in their smaller population (483 participants), consisting only of men. Extrapolating this value to 1995 USA population data, this represents 60,000 new cases of neovascular AMD per year (Table 6).[148] This lower value may be a reflection of a less reliable smaller data set as well as its lack of incidence data from women. In the Chesapeake Bay group, the incidence of neovascular AMD was higher in an older age group (2.0% 5-year incidence in a group aged 70–95 years) similar to the Beaver Dam study (3.2% 5-year incidence in a group aged 75–84 years).[14,81] Notably, half of the Chesapeake Bay study participants were less than 50 years old, which represents mostly a younger population in which AMD is less common. Their study does not represent a cross-sectional study of a general population, and many of the older participants in the initial prevalence study were excluded from the 5-year incidence study because they had died or were too weak to have fundus photographs taken. Therefore, the reported incidence rates from the Chesapeake Bay study are likely to be lower than the actual incidence of neovascular AMD.

TABLE 6

Estimates of the Prevalence and Incidence of Choroidal Neovascularization Secondary to AMD in the USA

Source of Data and Date of Study	Population Age Group (years)	Prevalence of CNV (%)	Five-year Incidence of CNV (%)	Estimated Cases of CNV using 1995 USA Population Data
Beaver Dam, WI, 1992[82]	43–54	0.1*		39,000
	55–64	0.5*		110,000
	65–74	1.0*		190,000
	75–86	5.2		580,000
	43–86 (total)	1.2		1,100,000
Chesapeake Bay, MD, 1989[13]	50–95	1.4*		960,000
	70–95	4.5*		1,100,000
	30–95 (total)	0.5		750,000
Vision Problems in United States, 1994[146]	40 + (only whites)			920,000*†
Framingham, MA, 1980[96]	52–64	0.08		23,000
	65–74	0.8		150,000
	75–85	4.00		460,000
	52–85 (total)	1.50		890,000
Iceland, 1987[68]	43–62	0.0		0
	63–72	1.3		250,000
	73–82	4.3		560,000
	83+	16.3		960,000
	43+ (total)‡			1,770,000
Rotterdam, the Netherlands, 1995[153]	55–64	0.1		21,000
	65–74	0.4		75,000
	75–84	2.4		270,000
	85–98	7.4		270,000
	55–98 (total)	1.1		600,000
Beaver Dam, WI, 1997[81]	75–86		3.2	71,000 new cases/year
	43–86 (total)		0.6	110,000 new cases/year
Chesapeake Bay, MD, 1995[14]	70–95		2.0	97,000 new cases/year
	30–95 (total)		0.2	60,000 new cases/year

Reported prevalence and incidence data were applied to 1995 USA population data to calculate estimates of numbers of cases in the USA.[148] AMD = age-related macular degeneration; CNV = choroidal neovascularization.

*Based on the finding by Klein et al that neovascular changes are present in three-fourths of late-stage AMD cases, the reported values on the prevalence of late-stage AMD that included nonneovascular atrophic changes were adjusted by three-fourths to estimate the prevalence rate of only exudative/neovascular AMD.[82]

†This reported value was based on 1990 USA population data and the Beaver Dam Eye Study.[82,146]

‡Because no prevalence data were reported by this study for the total population (aged 43 years and above), this estimate of the total number of cases of neovascular AMD was calculated by summing the estimated cases from each of the age categories that composed the entire population (aged 43 years and above).

In addition to establishing the prevalence and incidence of neovascular AMD, many epidemiologic studies have focused on identifying risk factors for all types of ARM and for the neovascular late-stage AMD. The following characteristics have been associated with an increased risk of early AMD: white race, female sex, low intake of antioxidants and other micronutrients, systemic hypertension, cardiovascular disease, and cigarette smoking.[28,49,64,69,80,81,83,104,109,136] For CNV in AMD, an elevated risk has been associated with the following characteristics: hyperopia, high levels of light exposure, light iris color, and particular features of macular drusen and the RPE.[15,19,26,37,38,45,54,55,61,130,139,145] These features include a greater number of drusen, large drusen, confluent drusen, soft drusen, and focal hyperpigmentation of the RPE.[100]

The Macular Photocoagulation Study Group has quantified risk factors for the development of CNV in a multicenter study with 670 patients.[100] All of the enrolled patients had CNV secondary to AMD in one eye, and the study examined the characteristics of the unaffected eye and other systemic factors to determine those associated with the development of CNV in the fellow eye. The following factors were associated with an increased risk of developing CNV: presence of five or more drusen (relative risk, 2.1), focal hyperpigmentation (relative risk, 2.0), one or more large drusen (relative risk, 1.5), and definite systemic hypertension (relative risk, 1.7).[100] These results are consistent with the Beaver Dam Eye Study finding that neovascular AMD is more likely to develop in eyes with retinal pigment abnormalities at

baseline than in eyes without these lesions; this study also showed a higher incidence of neovascular AMD in eyes with soft indistinct drusen at baseline.[81]

Many groups have documented the prevalence and incidence of neovascular AMD. In addition, extensive descriptions of CNV secondary to AMD and its associated risk factors have been reported. However, much remains to be discovered concerning the cause of the development and progression of these neovascular changes. The current and future epidemiologic studies and research efforts will hopefully lead to a better understanding of the pathogenesis of these changes and ultimately to a cure for AMD.

V. Treatment

The current treatment for many forms of ocular neovascularization involves photocoagulation or cryotherapy to either the ischemic retina, as in the cases of proliferative diabetic retinopathy, ROP, and CRVO, or to the neovascularization itself in the case of CNV. A review of the data, however, shows that for AMD, in particular, efficacy is limited, and improved therapeutic measures are greatly needed. Results from treatment studies will be presented for diabetic retinopathy and CNV of AMD.

A. TREATMENT OF DIABETIC RETINOPATHY

The Diabetic Retinopathy Study Group conducted a randomized controlled clinical trial evaluating photocoagulation therapy for proliferative diabetic retinopathy.[31] Eligible patients for this study had proliferative retinopathy in at least one eye or severe nonproliferative retinopathy in two eyes and visual acuity of 20/100 or better in each eye. As described previously, severe nonproliferative retinopathy may be characterized by early vascular changes, IRMAs. Photocoagulation as used in this study reduced the risk of severe visual loss by 50% or more and inhibited the progression of retinopathy. However, harmful effects of therapy were also observed, such as loss of visual acuity, macular edema, and constriction of peripheral visual fields.[34] However, the risk of visual loss without therapy outweighed the risks with treatment in the two groups with more severe disease: 1) eyes with new vessels and preretinal or vitreous hemorrhage and 2) eyes with new vessels on or within 1 disk diameter of the optic disk.[34] In these high-risk groups, the frequency of developing severe visual loss without treatment is roughly 26% to 40%, which justifies the use of photocoagulation. The Early Treatment Diabetic Retinopathy Study has shown a statistically significant benefit of early photocoagulation in preventing severe visual loss, compared with deferring scatter photocoagulation until high-risk proliferative retinopathy develops.[35] Currently, for patients with severe nonproliferative diabetic retinopathy or proliferative retinopathy, prompt intervention with scatter photocoagulation and vitrectomy when necessary can lead to a best-case reduction of the 5-year risk of severe visual loss by as much as 90%.[39] However, even with such high reduction of severe visual loss in some certain instances, the current treatments are not optimal because they can be associated with significant side effects, such as decreased peripheral and night vision, iatrogenic complications (e.g., lenticular or foveal burns and vitreous hemorrhage), macular edema, and visual loss, despite timely and appropriate intervention.[2] Thus, there still exists a need for more efficacious, nondestructive therapies for retinal neovascularization secondary to diabetes, and pharmacologic therapies based on angiogenic growth factor research may prove beneficial in the future.

Current research efforts devoted to diabetic retinopathy have also focused on prevention as opposed to treatment. In a landmark randomized clinical trial, the Diabetes Control and Complications Trial Research Group studied the effect of intensive insulin therapy on the development and progression of diabetic retinopathy.[30] Intensive insulin therapy consisted of administration of insulin at least three times a day by injection or the placement of an insulin pump. In contrast, conventional treatment consisted of only one or two daily insulin injections. The development of retinopathy during 8 years in the primary cohort was 54.1% with conventional treatment compared with 11.5% with intensive treatment. Furthermore, the progression of retinopathy in the conventional treatment cohort was 49.2% as compared with 17.1% with intensive treatment. It was therefore recommended that patients with insulin-dependent diabetes mellitus use intensive glycemic control to prevent complications, such as retinopathy and progression to proliferative retinopathy.[30] This finding is consistent with another study that showed a possible reduction of developing retinopathy in insulin-dependent diabetic patients in whom excellent glycemic control was achieved from the time of diagnosis.[88] The optimal intervention to drastically decrease diabetic proliferative retinopathy may require the combination of both improved treatments and prevention measures.

B. TREATMENT OF AGE-RELATED MACULAR DEGENERATION

Choroidal neovascularization of AMD is amenable to laser photocoagulation for a limited number of patients who are eligible according to the guidelines of the Macular Photocoagulation Study Group.[98] Therapy is limited because many patients have poorly imaged, ill-defined or occult neovascular lesions. Freund et al found that 87% of 67 patients

with newly diagnosed neovascularization of AMD did not meet the eligibility criteria of the Macular Photocoagulation Study, illustrating the need for new techniques of diagnosis and treatment.[43]

Even in eligible and treated eyes, there is a significant degree of recurrence. The Macular Photocoagulation Group studied 247 eyes that were treated with laser photocoagulation.[98] In 32%, recurrence was detected within 6 weeks of therapy. Their study estimated a 47% recurrence of neovascularization within a 5-year period. With laser photocoagulation, healthy portions of the retina or choroid are damaged, and visual results after treatment can be very poor. The recurrence rates of neovascularization are also unacceptably high.

C. SUMMARY

Current treatments for ocular neovascularization, such as laser photocoagulation, have been shown to be therapeutic in some cases. However, many limitations have surfaced. First, not all eyes with neovascularization are considered eligible for therapy. For instance, 87% of CNV cases are not amenable to therapy.[43] Second, of treated eyes, a certain proportion will show recurrence of neovascularization. In 32% of eligible and treated eyes with prior CNV, new vessels were present within 6 months, and the 5-year incidence of recurrence was estimated at 47%.[98] Third, it is unclear whether additional photocoagulation is beneficial, as harmful effects begin to outweigh benefits.

Results on the prevention of diabetic retinopathy appear to be more promising. The Diabetes Control and Complications Trial showed a reduced risk of development and progression of retinopathy in diabetic patients with intensive insulin therapy.[30] Overall, more efficacious and nondestructive interventions are needed to limit the damaging effects of neovascularization throughout the eye and to ultimately prevent the development of these new vessels.

VI. Conclusions

Ocular neovascularization is a widespread destructive process that is involved in nearly all major eye diseases and causes of blindness. Included are many worldwide infections, CRVO, diabetic retinopathy, macular degeneration, and ROP. Ocular neovascularization spans a vast spectrum in geography, in age (affecting neonates to the elderly), in sex, and in range of disease occurring in local and systemic conditions. The belief and observation that neovascularization is widespread is substantiated with tangible statistics by the data presented in this study. In addition to being highly prevalent around the world, neovascularization in the eye causes extremely harmful effects to the affected individuals. The importance of discovering the incidence and prevalence of neovascularization is critical to undertake the appropriate planning for costs, for disease prognosis, and for hope of prevention.

Data primarily from the Diabetic Retinopathy Study and Macular Photocoagulation Study Groups as well as others show convincingly that new vessel growth is directly linked to visual loss in proliferative diabetic retinopathy and exudative AMD. These studies show that treatments that shrink or ablate new vessels lead to improved vision or the halted progression of declining vision. As shown in this article, the greatest amount of epidemiologic research, such as prevalence, incidence, and risk factor studies, has been done in these two specific conditions: diabetic retinopathy and AMD.

In other ocular diseases, the direct effect of neovascularization is less clear. Research continues to be active as the story of neovascularization is still unfolding and evolving. More extensive epidemiologic studies are greatly needed in other areas, such as neovascularization of the cornea with contact lens usage, of the iris with CRVO, of the retina with sickle cell disorders, or of the choroid with ocular histoplasmosis syndrome. The high prevalence of neovascularization throughout the eye as outlined in this study underscores the potential enormous impact that future epidemiologic studies will have in uncovering risk factors or predisposing conditions that will lead toward a better understanding of the pathophysiology and ultimately toward more effective treatments.

An understanding of neovascularization in the eye provides a model for understanding the process of angiogenesis in general. Neovascularization in the eye can be easily detected through ophthalmologic examinations, and it reveals principles of angiogenesis that can be applied to other parts of the body. In summary, learning how to control ocular neovascularization offers the potential to achieve control over many of the major causes of visual loss and blindness in the world.

Method of Literature Search

Search terms included various combinations of the terms listed in the "Key words" section of this article as well as the words *cornea, iris, retina, and choroid.* A MEDLINE search was performed for publications between 1966 and the present. Articles, monographs, and book chapters obtained from the reference lists of other articles were reviewed and included when considered appropriate. Criteria for inclusion included the present epidemiologic value or the original importance of the article to a particular subject. Primary authors were also consulted.

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Dramatic Inhibition of Retinal and Choroidal Neovascularization by Oral Administration of a Kinase Inhibitor

Man Seong Seo,* Nohoon Kwak,* Hiroaki Ozaki,* Haruhiko Yamada,* Naoyuki Okamoto,* Eri Yamada,* Doriano Fabbro,† Francesco Hofmann,† Jeanette M. Wood,† and Peter A. Campochiaro*

From the Departments of Ophthalmology and Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland, and the Division of Oncology Research,† Pharmaceutical Division, Research and Development, Novartis Ltd. Pharmaceuticals, Inc., Basel, Switzerland*

The most common cause of new blindness in young patients is retinal neovascularization, and in the elderly is choroidal neovascularization. Therefore, there has been a great deal of attention focused on the development of new treatments for these disease processes. Previous studies have demonstrated partial inhibition of retinal neovascularization in animal models using antagonists of vascular endothelial growth factor or other signaling molecules implicated in the angiogenesis cascade. These studies have indicated potential for drug treatment, but have left many questions unanswered. Is it possible to completely inhibit retinal neovascularization using drug treatment with a mode of administration that is feasible to use in patients? Do agents that inhibit retinal neovascularization have any effect on choroidal neovascularization? In this study, we demonstrate complete inhibition of retinal neovascularization in mice with oxygen-induced ischemic retinopathy by oral administration of a partially selective kinase inhibitor that blocks several members of the protein kinase C family, along with vascular endothelial growth factor and platelet-derived growth factor receptor tyrosine kinases. The drug also blocks normal vascularization of the retina during development but has no identifiable adverse effects on mature retinal vessels. In addition, the kinase inhibitor causes dramatic inhibition of choroidal neovascularization in a laser-induced murine model. These data provide proof of concept that pharmacological treatment is a viable approach for therapy of both retinal and choroidal neovascularization. ***(Am J Pathol 1999, 154:1743–1753)***

The retina receives its blood supply from two vascular beds: retinal vessels, which supply the inner two-thirds of the retina, and choroidal vessels, which supply the outer one-third. Damage to retinal blood vessels resulting in closure of retinal capillaries and retinal ischemia occurs in several disease processes, including diabetic retinopathy, retinopathy of prematurity, branch retinal vein occlusion, and central retinal vein occlusion; they are collectively referred to as ischemic retinopathies. Retinal ischemia results in release of one or more angiogenic factors that stimulate neovascularization. The new vessels break through the internal limiting membrane that lines the inner surface of the retina and grow along the outer surface of the vitreous. They recruit many other cells and produce sheets of vessels, cells, and extracellular matrix that exert traction on the retina, often leading to retinal detachment and severe loss of vision. Panretinal laser photocoagulation increases oxygenation in the retina and can result in involution of neovascularization.[1] However, despite the effectiveness of laser photocoagulation,[2] diabetic retinopathy remains the most common cause of severe vision loss in patients less than 60 years

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M.S. Seo and N. Kwak contributed equally to the manuscript.

M.S. Seo's current address: Chonnam National University Medical School and Hospital, Kwangju, Korea.

N. Kwak's current address: Department of Ophthalmology, the Catholic University of Korea School of Medicine.

H. Ozaki's current address: Fukuoka University School of Medicine, Fukuoka, Japan.

Address reprint requests to Dr. Peter A. Campochiaro, Maumenee 719, The Johns Hopkins University School of Medicine, 600 N. Wolfe Street, Baltimore, MD 21287-9277. E-mail: pcampo@jhmi.edu.

of age in developed countries, and therefore additional treatments are needed.

Choroidal neovascularization occurs in several diseases in which there are abnormalities of Bruch's membrane. The most prevalent disease of this type is age-related macular degeneration, the most common cause of severe vision loss in patients over the age of 60 in developed countries.[3] Neovascularization originating from choroidal vessels grows through Bruch's membrane into the sub-retinal pigmented epithelial space and sometimes into the subretinal space. The blood vessels leak fluid, which collects beneath the retina causing reversible visual loss, and they bleed and cause scarring that results in permanent loss of central vision. Current treatments are designed to destroy or remove the abnormal blood vessels and do not address the underlying stimuli responsible for neovascularization; therefore, recurrent neovascularization and permanent visual loss occur in the majority of patients who initially have successful treatment.[3] Drug treatment that blocks the stimuli for choroidal neovascularization would be a major advance, but its development is hindered by our poor understanding of pathogenesis.

More is known about the cascade of events leading to retinal neovascularization than that for choroidal neovascularization, because some of the molecular signals involved in the development of retinal neovascularization have been defined. For instance, several lines of evidence suggest that vascular endothelial growth factor (VEGF) plays an important role in retinal vascularization during development and in pathological neovascularization in ischemic retinopathies. The expression of VEGF is increased by hypoxia,[4,5] which is a prominent feature of both of these processes. Stimulated by VEGF released by the avascular, hypoxic peripheral retina, blood vessels begin to develop at the optic nerve and extend to the periphery of the retina.[6] Likewise, VEGF participates in pathological retinal neovascularization, because its levels are increased in the retina and vitreous of patients[7–10] or laboratory animals[11,12] with ischemic retinopathies, and increased expression of VEGF in retinal photoreceptors of transgenic mice stimulates neovascularization within the retina.[13] The implication of VEGF in retinal neovascularization led to studies investigating VEGF antagonists in models of ischemic retinopathy. Soluble VEGF receptor/IgG fusion proteins or VEGF antisense oligonucleotides each inhibited retinal neovascularization by ~50% in the murine model of oxygen-induced ischemic retinopathy.[14,15] Antibodies to VEGF partially inhibited iris neovascularization in a monkey model of ischemic retinopathy.[16]

Although VEGF plays a central role, it is not the only stimulator involved, which might explain why VEGF antagonists are only partially effective. Growth hormone acting through insulin-like growth factor (IGF)-I also participates in retinal neovascularization, and decreased IGF-I in genetically engineered mice or antagonism of IGF-I by somatostatin analogs results in approximately a 30% decrease in retinal neovascularization in mice with ischemic retinopathy.[17]

Intracellular signaling induced by VEGF is complex, but it has been suggested that protein kinase C (PKC), particularly the PKCβII isoform, plays a prominent role.[18,19] A specific antagonist of PKCβ isoforms partially inhibits retinal neovascularization after laser-induced branch vein occlusion.[20]

Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are induced on endothelial cells, including those in the retina, participating in neovascularization.[21,22] Two independent studies using different peptides that antagonize binding to $\alpha_v\beta_3$ or both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ each demonstrated up to 50% inhibition of retinal neovascularization in the murine model of oxygen-induced ischemic retinopathy.[22,23]

Thus, several different types of agents that work by different mechanisms can cause partial inhibition of retinal neovascularization. This suggests that drug treatment of retinal neovascularization in patients may be feasible, but there are several questions related to this issue that remain. For instance, why is it that 50% inhibition has been the maximal achievable limit with several types of agents given by different routes of administration, including intraocular injections? Is there so much redundancy built into the retinal neovascularization cascade that this is the most that can be attained, and if so, is it sufficient to provide clinical benefit? Would an agent or combination of agents that act on multiple targets in the cascade be more effective? Do any of the molecular signals implicated in retinal neovascularization also play a role in choroidal neovascularization, and are there agents that inhibit both?

PKC consists of a family of at least 10 related serine/threonine kinases.[24] Staurosporine is an alkaloid produced by bacteria that is a potent nonspecific inhibitor of PKC[25] that also inhibits other serine/threonine kinases, such as protein kinase A (PKA), and tyrosine kinases, such as epidermal growth factor receptor (EGFR).[26] CGP 41251 is a derivative of staurosporine with the chemical name *N*-benzoyl-staurosporine that was developed as a PKC inhibitor for treatment of cancer.[26] It is a less potent inhibitor of PKC than staurosporine but is more specific because it is a weak inhibitor of PKA and EGFR, and it has been used in several studies to assess the role of PKC in cellular functions.[27–30] Recently, one of the authors (J.M. Wood) determined that CGP 41251 is also a relatively potent inhibitor of VEGF and platelet-derived growth factor (PDGF) receptor tyrosine kinases (unpublished data). It was also shown to inhibit VEGF-induced angiogenesis in a mouse subcutaneous growth factor implant model. As antagonism of VEGF and inhibition of PKC, each have been demonstrated to partially inhibit retinal neovascularization[14,15,20] and CGP 41251 has both activities, we investigated the effect of CGP 41251 in animal models of retinal and choroidal neovascularization.

Materials and Methods

Measurement of Inhibitory Activity of CGP 41251 in Vitro

The effect of CGP 41251 on the enzymatic activity of several members of the PKC family was measured using purified enzymes and artificial substrates as previously

described.[29] The effect of CGP 41251 on phosphorylation of VEGFRs and other tyrosine kinase receptors was measured using purified recombinant glutathione S-transferase (GST)-fused kinase domains in the presence of substrate and labeled ATP.[31] The kinase domain-fusion proteins were expressed in baculovirus, purified over glutathione-Sepharose, and diluted in 10 mmol/L Tris/HCl (pH 7.2) based on their specific activity to obtain an activity of 4000 to 6000 cpm above background (<400 cpm). [^{33}P]ATP (Amersham, Arlington Heights, IL) was used as the phosphate donor, and the polyGlu-Tyr(4:1) peptide (P-275, Sigma Chemical Co., St. Louis, MO) was used as the acceptor. CGP 41251 was dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mmol/L and then diluted as required so that the final DMSO concentration was 1%. The assay mixture, which was optimized for each kinase (20 mmol/L Tris/HCl (pH 7.5), 1 to 10 mmol/L $MnCl_2$, 1 to 10 mmol/L $MgCl_2$, 1 to 8 μmol/L ATP, 0.2 μCi of [^{33}P]ATP, 3 to 8 μg/ml polyGlu-Tyr(4:1)) was incubated with the respective GST-fused kinase with or without CGP 41251 for 10 minutes at room temperature in a total volume of 30 μl. The reaction was stopped by adding 10 μl of 250 mmol/L EDTA. Using a 96-well filter system (GIBCO BRL, Gaithersburg, MD), 20 μl of the reaction mixture was transferred onto an Immobilon-PVDF membrane (IPVH 000 10, Millipore, Bedford, MA). Membranes were washed extensively with 0.5% H_3PO_4 and soaked in ethanol. After drying, Microscint cocktail (TM-0 6013611, Packard, Meriden, CT) was added, and scintillation counting was performed (Hewlett Packard Top Count). IC50 values were calculated by linear regression analysis of the percentage inhibition by CGP 41251 over a range of different concentrations

Drug Treatment of Mice with Ischemic Retinopathy

Ischemic retinopathy was produced in C57BL/6J mice by a method described by Smith et al.[32] Seven-day-old (P7) mice and their mothers were placed in an airtight incubator and exposed to an atmosphere of 75 ± 3% oxygen for 5 days. Incubator temperature was maintained at 23 ± 2°C, and oxygen was measured every 8 hours with an oxygen analyzer. After 5 days, the mice were removed from the incubator and placed in room air, and drug treatment was begun. Drug was dissolved in DMSO and diluted to final concentrations with water; the maximal concentration of DMSO was 1%. Vehicle (1% DMSO) or vehicle containing various concentrations of drug (volume = 10 μl per gram body weight) was placed in the stomach by gavage once a day. At P17, after 5 days of treatment, mice were sacrificed, and eyes were rapidly removed and frozen in optimal cutting temperature embedding compound (OCT; Miles Diagnostics, Elkhart, IN) or fixed in 10% phosphate-buffered formalin and embedded in paraffin. Adult C57BL/6J mice were also treated by gavage with drug or vehicle, and after 5 days, they were sacrificed and their eyes were processed for frozen or paraffin sections.

Quantitation of Retinal Neovascularization

Frozen sections (10 μm) of eyes from drug-treated and control mice were histochemically stained with biotinylated griffonia simplicifolia lectin B4 (GSA, Vector Laboratories, Burlingame, CA), which selectively binds to endothelial cells. Slides were incubated in methanol/H_2O_2 for 10 minutes at 4°C, washed with 0.05 mol/L Tris-buffered saline, pH 7.6 (TBS), and incubated for 30 minutes in 10% normal porcine serum. Slides were incubated for 2 hours at room temperature with biotinylated GSA, and after rinsing with 0.05 mol/L TBS, they were incubated with avidin coupled to peroxidase (Vector Laboratories) for 45 minutes at room temperature. After being washed for 10 minutes with 0.05 mol/L TBS, slides were incubated with diaminobenzidine to give a brown reaction product. Some slides were counterstained with hematoxylin, and all were mounted with Cytoseal.

To perform quantitative assessments, 10-μm serial sections were cut through one-half of each eye, and sections roughly 50 to 60 μm apart were stained with GSA, providing 13 sections per eye for analysis. GSA-stained sections were examined with an Axioskop microscope (Zeiss, Thornwood, NY), and images were digitized using a 3 CCD color video camera (IK-TU40A, Toshiba, Tokyo, Japan) and a frame grabber. Image-Pro Plus software (Media Cybernetics, Silver Springs, MD) was used to delineate GSA-stained cells on the surface of the retina, and their area was measured. The mean of the 13 measurements from each eye was used as a single experimental value.

Drug Treatment of Mice during Retinal Vascular Development

Litters of newborn C57BL/6J mice were divided into treatment and control groups that received daily subcutaneous injections of 100 mg/kg drug or vehicle, respectively. At P7 or P10, mice were anesthetized and perfused with 1 ml of phosphate-buffered saline containing 50 mg/ml fluorescein-labeled dextran (2×10^6 average molecular weight; Sigma) as previously described.[33] The eyes were removed and fixed for 1 hour in 10% phosphate-buffered formalin. The cornea and lens were removed and the entire retina was carefully dissected from the eyecup. Radial cuts were made from the edge of the retina to the equator in all four quadrants, and the retina was flat mounted in Aquamount with photoreceptors facing upward. Flat mounts were examined by fluorescence microscopy, and images were digitized using a 3 CCD color video camera and a frame grabber. Image-Pro Plus was used to measure the distance from the center of the optic nerve to the leading front of developing retinal vessels in each quadrant, and the mean was used as a single experimental value.

Drug Treatment of Mice with Laser-Induced Choroidal Neovascularization

Choroidal neovascularization was generated by modification of a previously described technique.[34] Briefly, 4- to 5-week-old male C57BL/6J mice were anesthetized with ketamine hydrochloride (100 mg/kg body weight), and the pupils were dilated with 1% tropicamide. Three burns of krypton laser photocoagulation (100-μm spot size, 0.1-second duration, 150 mW) were delivered to each retina using the slit lamp delivery system of a Coherent model 920 photocoagulator and a hand-held cover slide as a contact lens. Burns were performed in the 9, 12, and 3 o'clock positions of the posterior pole of the retina. Production of a bubble at the time of laser, which indicates rupture of Bruch's membrane, is an important factor in obtaining choroidal neovascularization,[34] so only mice in which a bubble was produced for all three burns were included in the study. Ten mice were randomly assigned to treatment with vehicle alone, and 10 mice received vehicle containing 400 mg/kg/day of CGP 41251 orally by gavage. After 14 days, the mice were killed with an overdose of pentobarbital sodium, and their eyes were rapidly removed and frozen in OCT.

Quantitative Analysis of the Amount of Choroidal Neovascularization

Frozen serial sections (10 μm) were cut through the entire extent of each burn and histochemically stained with biotinylated GSA as described above. Histomark Red (Kirkegaard and Perry, Gaithersburg, MD) was used as chromogen to give a red reaction product that is distinguishable from melanin. Some slides were counterstained with Contrast Blue (Kirkegaard and Perry).

To perform quantitative assessments, GSA-stained sections were examined with an Axioskop microscope, and images were digitized using a 3 CCD color video camera and a frame grabber. Image-Pro Plus software was used to delineate and measure the area of GSA-stained blood vessels in the subretinal space. For each lesion, area measurements were made for all sections on which some of the lesion appeared and added together to give the integrated area measurement. Only lesions in which good sections were obtained through the entire lesion, so that a valid area measurement could be made on each, were included in the analysis. There appeared to be little variability among lesions in individual mice, and all excluded lesions were qualitatively similar in size to included lesions and were excluded solely due to inability to obtain an accurate measurement because of poor quality of some sections. Values were averaged to give one experimental value per mouse. A two-sample *t*-test for unequal variances was performed to compare the log mean integrated area between treated and control mice.

Table 1. Kinase Inhibition Profile of CGP 41251

Kinase	IC50 (μmol/L)
PKCα	0.022 ± 0.008
PKCβI	0.030 ± 0.018
PKCβII	0.031 ± 0.016
PKCγ	0.024 ± 0.006
PKCδ	0.33 ± 0.071
PKCζ	465 ± 49
PKCε	1.25 ± 1.06
PKCη	0.16 ± 0.095
KDR	0.086 ± 0.04
Flt-1	0.912 ± 0.244
Flk-1	1.013 ± 0.261
PDGFR-β	0.02
Tie2	>10.00
FGFR1	>10.00
EGFR	3.0[25]
PKA	2.4[25]

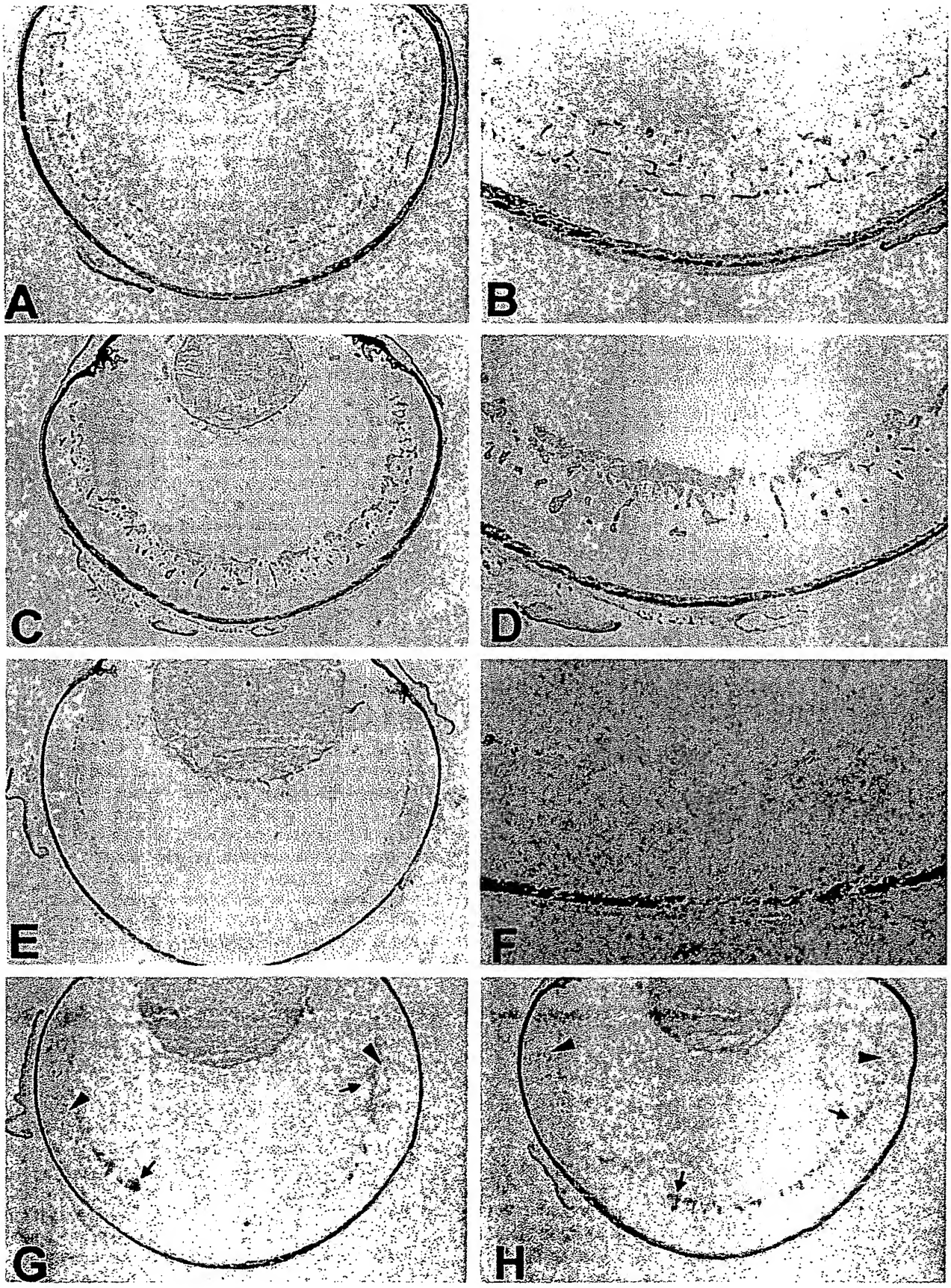
Each value represents the mean ± SEM calculated from at least three experimental values from at least two independent experiments.

Results

Characterization of in Vitro *Activity of CGP 41251*

Table 1 shows the kinase inhibitory profile of CGP 41251. The IC50 for several subtypes of PKC as well as the KDR tyrosine kinase of human VEGF receptor-2 and the tyrosine kinase of human PDGF receptor-β are in the same range (20 to 100 nmol/L). At approximately 10-fold higher concentrations, CGP 41251 inhibits Flk-1, the tyrosine kinase of the mouse VEGF receptor corresponding to human KDR, and Flt-1, the tyrosine kinase of human VEGF receptor-1. The IC50s for other receptor tyrosine kinases, such as Tie 2, fibroblast growth factor receptor-1, or epidermal growth factor receptor, are 3 μmol/L or above.

Figure 1. Treatment with CGP 41251 blocks retinal neovascularization in a dose-dependent manner in mice with ischemic retinopathy. P7 mice were put in high oxygen for 5 days and then removed to room air and given CGP 41251 or vehicle by gavage for 5 days. Retinal frozen sections were histochemically stained with the endothelial cell-selective lectin griffonia simplicifolia I using the peroxidase-antiperoxidase technique. Retinal blood vessels within the retina and neovascularization on the surface are stained with reaction product. **A:** Normal retinal vessels in a nonischemic P17 mouse. **B:** High magnification of normal retinal vessels in P17 mouse. **C:** A P17 mouse with ischemic retinopathy treated by gavage for 5 days with vehicle alone shows extensive neovascularization on the surface of the retina. **D:** High magnification of retina from **C** showing prominent neovascularization. **E:** A P17 mouse with ischemic retinopathy treated by gavage with 600 mg/kg CGP 41251 once a day for 5 days shows essentially complete absence of endothelial cells in the posterior retina, and there are only a few clumps of endothelial cells in the superficial part of the anterior retina with striking absence of the deep retinal capillary bed. **F:** High magnification of the anterior retina from **E** shows only a few clumps of endothelial cells in the superficial part of the retina with no neovascularization on the surface and no deep capillaries. **G:** A P17 mouse with ischemic retinopathy treated by gavage with 300 mg/kg CGP 41251 once a day for 5 days shows essentially complete absence of endothelial cells in the posterior retina, but in midperipheral and peripheral retina, there are retinal vessels and a few patches of neovascularization (**arrows**). The deep capillary bed is present in the periphery of the retina (**arrowheads**). **H:** A P17 mouse with ischemic retinopathy treated by gavage with 60 mg/kg CGP 41251 once a day for 5 days shows retinal vessels throughout the anterior and posterior retina, but the deep capillary bed is more evident in the periphery (**arrowheads**). Small patches of neovascularization are also seen on the surface of the posterior and anterior retina (**arrows**).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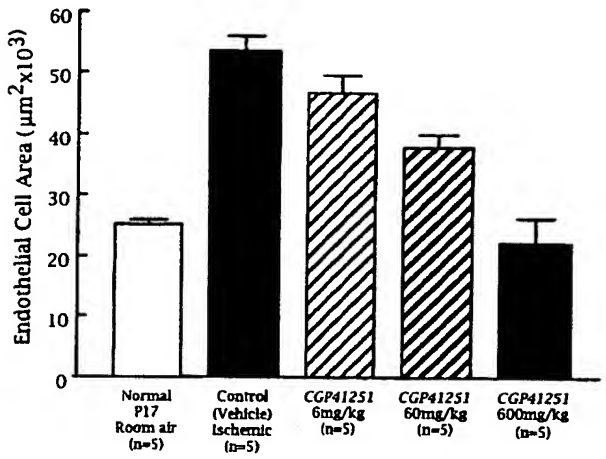

Figure 2. Quantitation of the area of endothelial cell staining in retinal sections of mice with ischemic retinopathy treated for 5 days by gavage with vehicle or vehicle containing various concentrations of CGP 41251. Total area of endothelial cell staining in retinal sections was determined by image analysis as described in Materials and Methods. There was a statistically significant difference between the control group and the three treated groups ($P < 0.001$ by ANOVA).

CGP 41251 Inhibits Retinal Neovascularization in Mice with Ischemic Retinopathy

Retinas of nonischemic P17 mice stained with GSA show normal vessels in the superficial and deep capillary beds with a few connecting vessels (Figure 1, A and B). P17 mice with ischemic retinopathy treated with vehicle show a marked increase in the area of endothelial cell staining throughout the retina with large clumps of cells on the retinal surface (Figure 1, C and D), not seen in nonischemic retinas. P17 mice with ischemic retinopathy given 600 mg/kg CGP 41251 once a day for 5 days by gavage have a dramatic decrease in endothelial cell staining on the surface and within the retina (Figure 1, E and F) compared with vehicle-treated mice. In fact, the endothelial cell staining within the retina is less than that seen in nonischemic P17 mice. High magnification shows that there are no identifiable endothelial cells on the surface of the retina, indicating that there is complete inhibition of neovascularization (Figure 1F). There is also a striking absence of endothelial cell staining in the inner nuclear layer and outer plexiform layer where the deep capillary beds are normally located. P17 mice with ischemic retinopathy given 300 mg/kg (Figure 1G) or 60 mg/kg (Figure 1H) CGP 41251 once a day by gavage show some clumps of neovascularization on the surface of the retina (arrows) that are less than those seen in vehicle-treated controls. They also show some decrease in endothelial staining within the retina, but there is some present (arrowheads). Image analysis demonstrates that mice treated once a day with 600, 60, or 6 mg/kg CGP 41251 show a statistically significant dose-dependent decrease in endothelial cell staining on and in the retinas compared with vehicle-treated mice ($P < 0.001$ by analysis of variance (ANOVA); Figure 2).

CGP 41251 Inhibits Retinal Vascular Development

The decrease in endothelial staining in the inner nuclear layer of the retinas of CGP-41251-treated mice suggests that there might be some inhibition of retinal vascular development, because the time of treatment (P12 to P17) corresponds to the period of development of the deep capillary bed.[35] To test this, neonatal mice were treated with subcutaneous injections of 100 mg/kg CGP 41251 or vehicle alone starting on P0, and on P7 or P10 they were perfused with fluorescein-labeled dextran and retinal whole mounts were prepared. At P7, retinal vessels in vehicle-treated mice have almost reached the peripheral edge of retina (Figure 3, A and C), but in CGP-41251-treated mice, the retinal vessels have extended only slightly more than halfway to the periphery (Figure 3, B and D). At P10, in vehicle-treated mice, the superficial capillary bed is complete and extends all of the way to the peripheral edge of the retina, and the deep capillary bed is partially developed. But in CGP-41251-treated mice, the superficial capillary bed has not yet reached the edge of the retina (not shown). The distance from the optic nerve to the vascular front was calculated by image analysis, and the differences between treated and control mice at P7 and P10 were statistically significant (Figure 4). This indicates that CGP 41251 inhibits normal retinal vascular development as well as pathological retinal neovascularization.

CGP 41251 Has No Identifiable Effect on Retinal Vessels in Adult Mice

Adult mice were given 600 mg/kg CGP 41251 by gavage once a day for 5 days, the highest dose used in the model of oxygen-induced ischemic retinopathy. There was no difference in the total area of endothelial staining in the retina or the appearance of retinal vessels in CGP-41251-treated mice compared with vehicle-treated mice (Figure 5, A and B). Image analysis shows no difference in the amount of retinal endothelial cell staining between CGP-41251- and vehicle-treated mice. This suggests that CGP 41251 is not toxic to endothelial cells of mature vessels.

CGP 41251 Inhibits Choroidal Neovascularization in Mice with Laser-Induced Rupture of Bruch's Membrane

Two weeks after laser photocoagulation, all lesions in both groups of mice showed a discontinuity in Bruch's membrane with roughly equivalent damage to the overlying retina. All mice treated with vehicle alone showed large areas of choroidal neovascularization at the site of each laser-induced rupture of Bruch's membrane (Figure 6, A and C). There was proliferation of retinal pigmented epithelial cells along the margin of the new vessels. Retinal blood vessels stained with GSA were seen in the overlying retina. In contrast, all mice given 400 mg/kg/

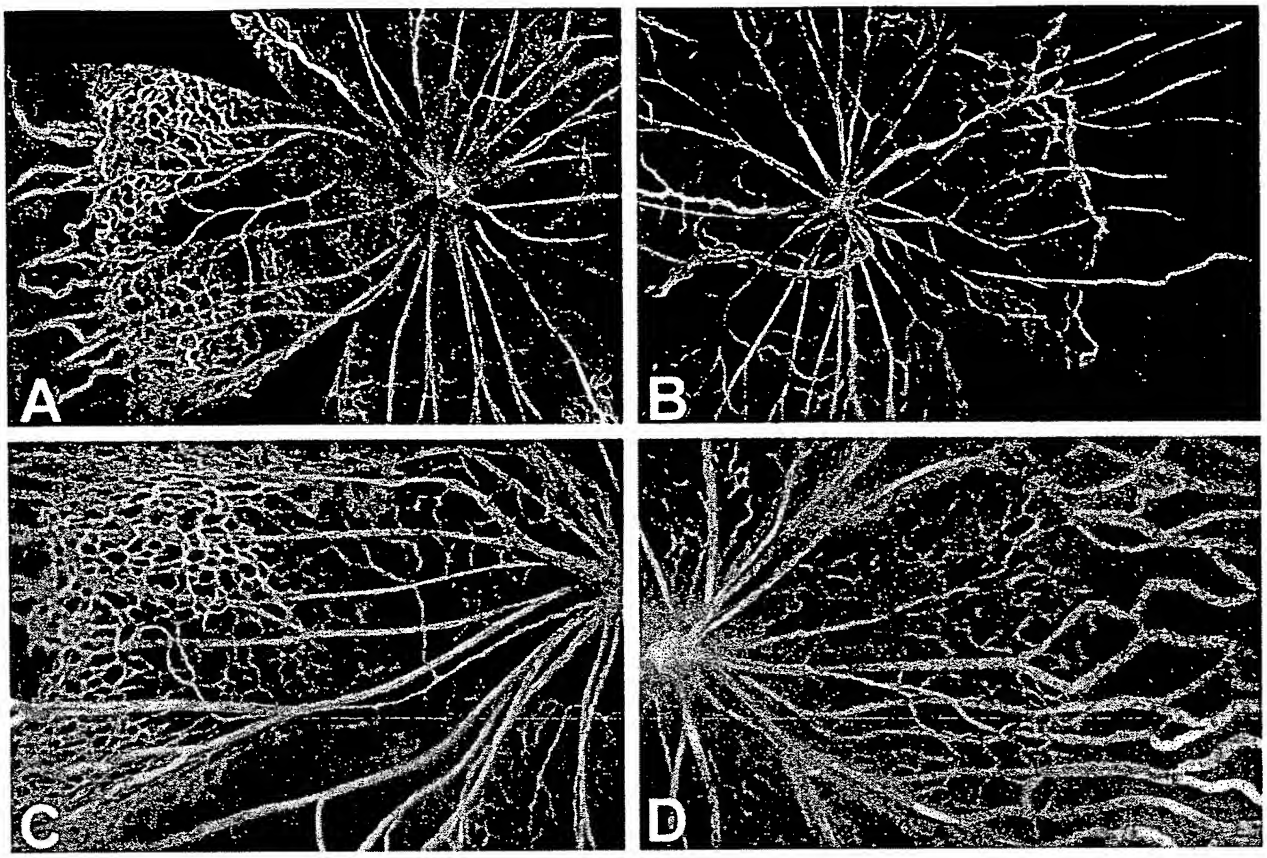

Figure 3. Treatment with CGP 41251 inhibits development of retinal blood vessels. Mice were given daily subcutaneous injections of vehicle (A and C) or vehicle containing 100 mg/kg CGP 41251 (B and D) starting on postnatal day 1 (P1) and were sacrificed on P7 by perfusion with fluorescein-labeled dextran. Fluorescence microscopy shows a leading front of developing retinal vessels extending further from the optic nerve in vehicle-treated retinas compared with retinas treated with CGP 41251. The large vessels are primary hyaloidal vessels that have not yet involuted. C: High magnification of control retina showing extensive development of retinal capillaries. D: High magnification of retina from CGP-41251-treated mouse showing marked inhibition of retinal capillary development.

day CGP 41251 by gavage had very little if any choroidal neovascularization at the site of each laser-induced rupture of Bruch's membrane. In many instances, there was no identifiable GSA-stained neovascular tissue throughout the entire burn (Figure 6D), but some burns contained regions in which there were thin disks of GSA-stained tissue (Figure 6B). There was mild proliferation of retinal pigmented epithelial cells. Despite the marked decrease in choroidal neovascularization in the eyes of treated mice, the overlying retinal vessels appeared normal. This was best seen in sections with no counterstain (Figure 6B).

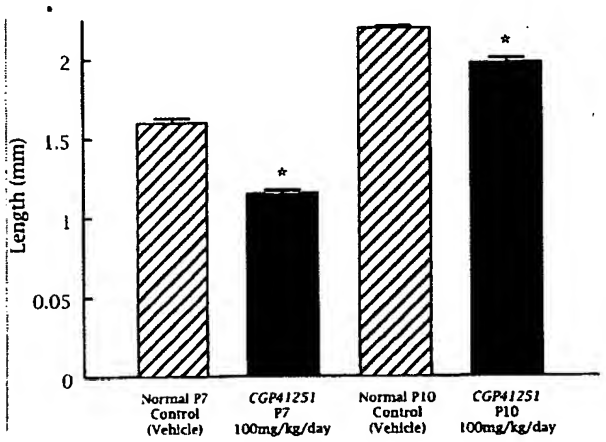

Figure 4. Quantitation of retinal vascular development in neonatal control and CGP 41251-treated mice. Retinal whole mounts were examined by fluorescence microscopy, and the distance from the optic nerve to the leading front of developing retinal capillaries was measured in all four quadrants to give an average value for each retina. For each group, $n = 5$. $P < 0.001$ for a dose-dependent decrease in CGP-41251-treated mice compared with age-matched controls by ANOVA.

Quantitation of the integrated area of GSA staining per lesion showed a dramatic decrease in mice treated with CGP 41251 compared with lesions in mice treated with vehicle alone. Table 2 shows the measurements for each of the lesions in which intact, adequately stained sections were obtained all of the way through the lesion, allowing measurement of the area of neovascularization on each section. In all but four mice, high quality, well stained inclusive sections were obtained for at least four of six lesions (two mice in the treated group and one in the control group had three measurable lesions, and one control mouse had two measurable lesions). Qualitatively, all of the lesions in an individual were very similar in size, with no systematic differences between included and excluded lesions except for the inability to obtain accurate quantitative assessment for excluded lesions. The mean of the measurements made in each individual

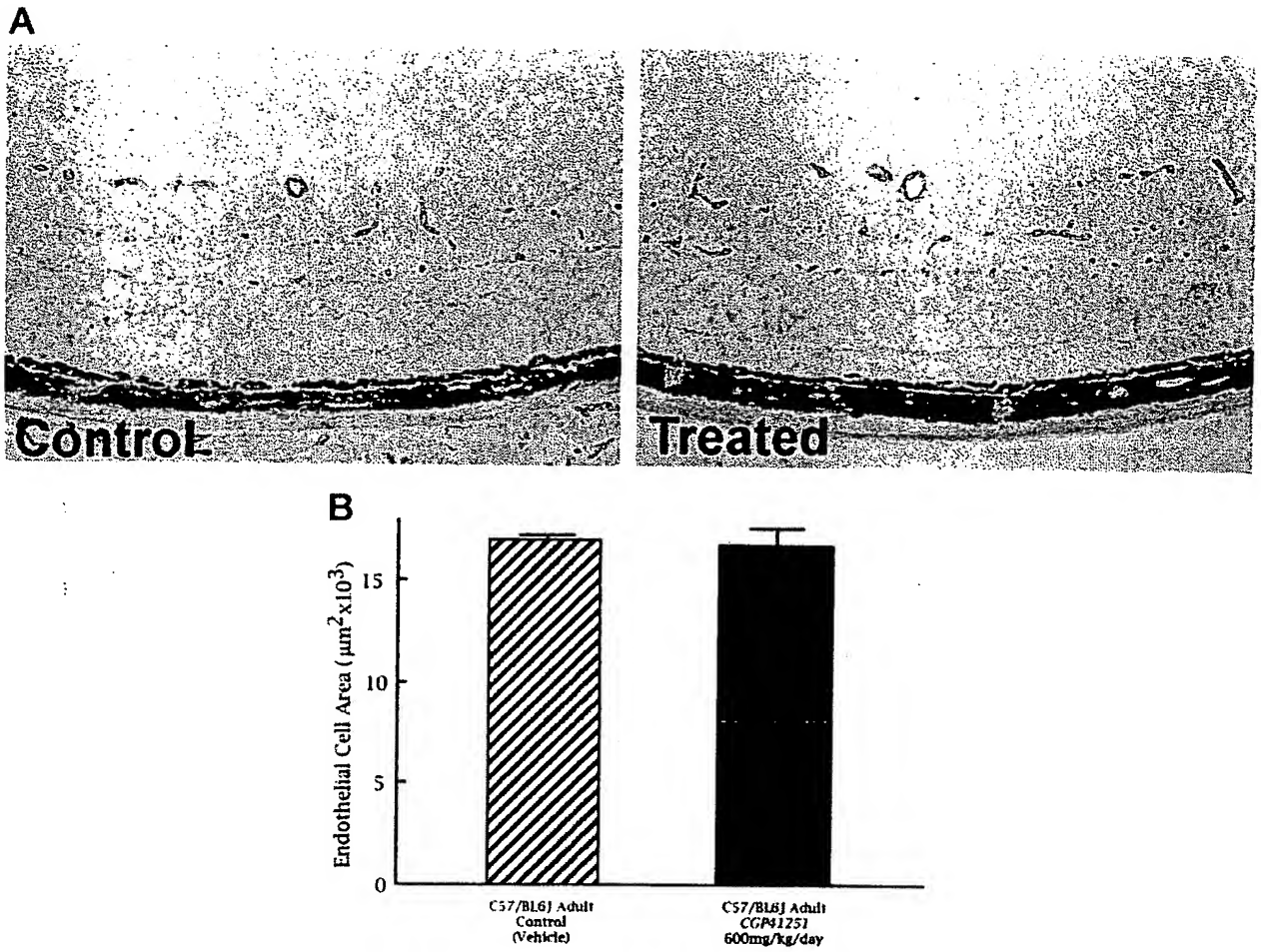

Figure 5. Treatment with CGP 41251 shows no identifiable toxicity to mature retinal blood vessels. **A:** Adult mice were treated by gavage for 5 days with vehicle or vehicle containing 600 mg/kg CGP 41251. Retinal frozen sections were histochemically stained with the endothelial-cell-selective lectin griffonia simplicifolia I using the peroxidase-antiperoxidase technique. There is no difference in the number or appearance of retinal blood vessels in treated versus control retinas. **B:** Quantitation of the total area of endothelial cell staining by image analysis shows no difference between control ($n = 5$) and CGP-41251-treated ($n = 5$) retinas ($P = 0.8489$ by paired *t*-test).

mouse constituted a single experimental value. The mean integrated area of choroidal neovascularization in 10 CGP 41251 mice was 0.0090182 ± 0.0017540 mm^2 compared with 0.0695621 ± 0.0073960 mm^2 in 10 control mice, nearly an eightfold difference. This difference was highly statistically significant ($P = 0.004$; Figure 7).

Discussion

Retinal neovascularization is a major cause of visual morbidity and blindness and often affects young people in their most productive years.[36] Although panretinal photocoagulation is clearly beneficial, there are many patients in whom it cannot be delivered or is not sufficient. Also, panretinal photocoagulation has side effects, including production or exacerbation of macular edema, decreased night vision, and decreased visual fields. For these reasons, a great deal of effort has been directed toward development of drug treatment for retinal neovascularization.

Previous studies have demonstrated that several pharmacological agents with different mechanisms of action are capable of partially inhibiting retinal neovascularization. Antagonism of VEGF by soluble VEGF receptors coupled to IgG heavy chains[14] or by antisense oligonucleotides[15] or blockade of integrin $\alpha_v\beta_3$ by two different cyclic peptides[22,23] each inhibit retinal neovascularization in the murine model of oxygen-induced ischemic retinopathy by as much as 50%. In this study, using the same model, we have demonstrated for the first time that it is possible to achieve essentially complete inhibition of retinal neovascularization by drug treatment. This provides strong support for the feasibility of using drugs to treat retinal neovascularization in patients with proliferative diabetic retinopathy or other blinding ischemic retinopathies. It is particularly encouraging that this dramatic treatment effect was achieved with oral administration, the preferred route of administration in patients.

The drug used in this study, CGP 41251, is an inhibitor of several PKC isoforms, including PKCβII, which has

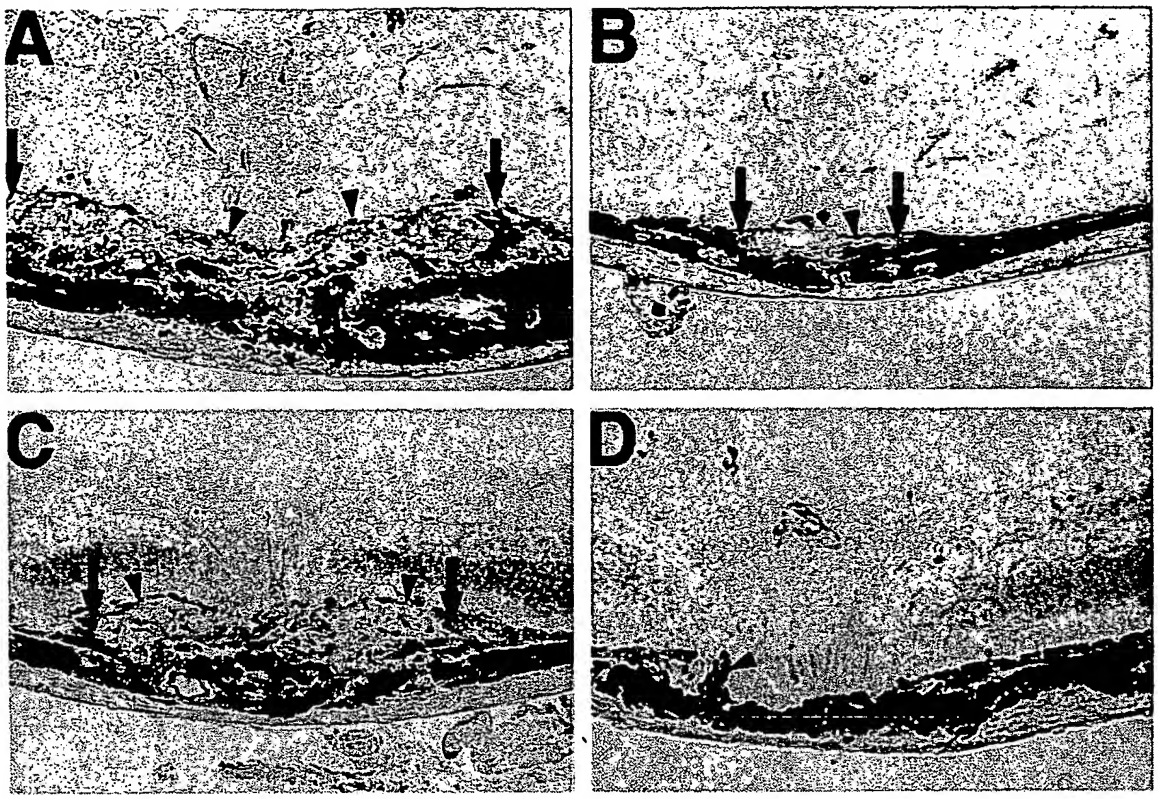

Figure 6. Oral administration of CGP 41251 inhibits the development of choroidal neovascularization. Mice given vehicle alone by gavage have extensive choroidal neovascularization 14 days after laser-induced rupture of Bruch's membrane. **A:** Histochemistry with griffonia simplicifolia lectin (GSA) and no counterstain shows a large area of staining in the subretinal space due to choroidal neovascularization and small focal areas of staining in the retina due to retinal blood vessels. **C:** A laser burn in another control mouse stained with GSA and counterstained with contrast blue shows prominent choroidal neovascularization. Mice given 400 mg/kg CGP 41251 have minimal choroidal neovascularization 14 days after laser-induced rupture of Bruch's membrane. **B:** GSA staining without counterstaining shows minimal choroidal neovascularization, and the overlying retinal vessels are normal in appearance. **D:** A laser burn in another CGP-41251-treated mouse shows essentially no choroidal neovascularization and normal retinal blood vessels in the overlying retina, which is counterstained with contrast blue.

been implicated in diabetic complications, including neovascularization in the retina.[19,20] CGP 41251 is also an inhibitor of phosphorylation by VEGF and PDGF receptors. As previous studies with agents that specifically block PKCβ isoforms or specifically antagonize VEGF have resulted in only partial inhibition of retinal neovascularization, it may be that the greater efficacy of CGP 41251 is due to an additive effect of these different activities. However, it is also possible that the inhibitory effect on retinal neovascularization occurs predominantly through one of these activities and the greater efficacy of CGP 41251 compared with the more specific agents is due to a difference in pharmacokinetics or a difference in mechanism of action. For instance, it could be that blockade of VEGF receptor phosphorylation is a more effective way to block VEGF signaling than attempting to limit the amount of VEGF available to bind to the receptor.[14,15] In any case, this study demonstrates that it is possible to achieve much better inhibition of retinal neovascularization by pharmacological means than was previously demonstrated, and additional work is needed to determine the mechanism through which this occurs so that additional effective drugs can be designed. Identification and testing of drugs with different kinase inhibitory activities may help to accomplish this goal.

Our data also indicate that CGP 41251 inhibits normal retinal vascular development in addition to pathological retinal neovascularization. Although we were surprised by this finding initially, because it was not reported to occur from treatment with other VEGF antagonists in the same model,[14,15] its occurrence is understandable, because VEGF has been demonstrated to be a critical stimulator of normal retinal vascular development.[6] Down-regulation of VEGF during development by hyperoxia arrests vascular development and causes vaso-occlusion.[37] When hyperoxia-induced blockade is followed by pharmacological blockade of VEGF signaling (as well as PDGF and PKC signaling), it is not surprising that parts of the retina never develop retinal vessels.

More is known regarding the molecular signals involved in retinal neovascularization than those involved in choroidal neovascularization. This and lack of an inexpensive animal model in which it is possible to precisely measure the amount of choroidal neovascularization have hindered identification of agents that inhibit choroidal neovascularization. We have recently adapted to mice[34] a model of laser-induced choroidal neovascularization that was first developed in primates.[38] In this study, we report a technique of quantitatively assessing the amount of choroidal neovascularization, providing a

Table 2. Integrated Area of Choroidal Neovascularization at Sites of Laser-Induced Rupture of Bruch's Membrane in CGP-41251-Treated and Control Mice

	Integrated area of measurable lesions	Average ($mm^2 \times 10^2$)
Treated mice		
1	1.34171, 2.01748, 1.97958	1.77959
2	1.28591, 0.57824, 1.13829, 0.81061, 1.19101	1.00081
3	1.50637, 0.79469, 0.50504, 0.46116	0.81681
4	1.54149, 1.09383, 0.92532, 1.71000	1.31766
5	0.11757, 0.05995, 0.04629, 0.05880	0.07065
6	0.73828, 1.21054, 1.00888, 0.81879, 1.15301	0.98590
7	0.87055, 0.90481, 2.91152, 1.12572, 1.99885	1.56229
8	1.59527, 0.29388, 1.46979, 0.08462, 0.38570	0.76585
9	0.07497, 1.04742, 0.84053, 0.20836	0.54282
10	0.24489, 0.10767, 0.17489	0.17581
Mean		0.90182
Control mice		
1	2.13169, 17.43091, 12.11940, 9.81230	10.37357
2	4.48336, 7.91262, 6.34613, 6.15369	6.22395
3	5.83853, 6.23603	6.03728
4	12.03223, 7.15583, 12.29029, 5.91466, 9.73981	9.42656
5	5.61468, 4.35111, 5.15342, 7.23744, 3.46248	5.16383
6	2.49107, 4.39638, 12.67973	6.52239
7	19.92445, 7.28564, 3.70144, 4.20451, 11.51673, 6.16789	8.80011
8	9.67250, 8.48616, 7.39480, 17.01920, 5.59292, 6.82986	9.16591
9	4.57032, 2.30611, 4.93298	3.93647
10	3.42368, 3.69508, 3.32311, 4.30905, 4.80916	3.91202
Mean		6.95621

means to objectively assess drug effects. Using this approach, we showed that oral administration of 400 mg/kg CGP 41251 dramatically inhibits choroidal neovascularization. This suggests that activation of PKC and/or VEGF signaling and/or PDGF signaling are involved in development of choroidal neovascularization in this model. By using drugs with different, but overlapping *in vitro* activities, it should be possible to further define the molecular signals involved in choroidal neovascularization.

The results of this study are encouraging in two other respects. CGP 41251 is the first drug identified to have a strong inhibitory effect on both retinal and choroidal neovascularization. Perhaps inhibitory activity in models of retinal neovascularization will have some predictive value for treatment of choroidal neovascularization. Additional correlative studies are needed to determine whether this is actually the case, but if so, it will simplify screening of drugs for ocular neovascularization. Second, although CGP 41251 is the first kinase inhibitor to be evaluated for its effect on retinal and choroidal neovascularization, and other agents in this class of drugs need to be evaluated and could possibly be more potent, the effects with CGP 41251 are dramatic and suggest that it may be useful for treatment of patients with retinal or choroidal neovascularization. The inhibitory effect of CGP 41521 on normal retinal vascular development may preclude its use in infants with retinopathy of prematurity, but the results of our study predict that CGP 41251 is a good candidate for treatment of adults with proliferative diabetic retinopathy and other ischemic retinopathies or choroidal neovascularization due to macular degeneration, ocular histoplasmosis, or a host of other diseases. Toxicity studies in animals have not identified any adverse systemic effects of orally administered CGP 41251 (unpublished data), and phase I clinical trials are underway in cancer patients. This study suggests that clinical trials in patients with retinal or choroidal neovascularization should also be considered.

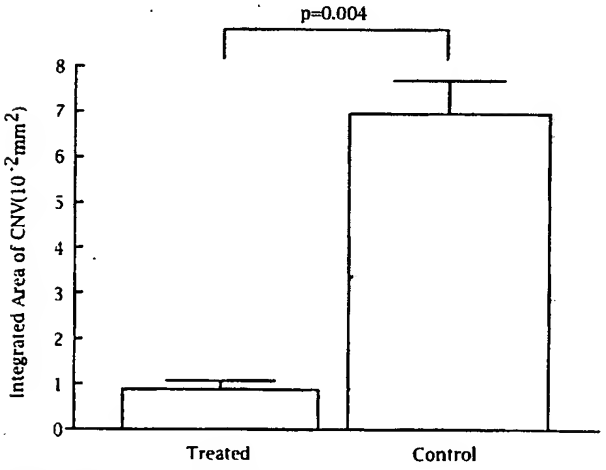

Figure 7. Quantitation of choroidal neovascularization in control and CGP-41251-treated mice with laser-induced rupture of Bruch's membrane shows a significantly smaller integrated area of choroidal neovascularization in CGP-41251-treated mice ($P = 0.004$ by two-sample *t*-test for unequal variances).

Acknowledgment

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Novel Approaches in the Treatment of Angiogenic Eye Disease

U. Wegewitz, I. Göhring and J. Spranger*

Department of Clinical Nutrition (Chairman Prof. Dr. A.F.H. Pfeiffer), German Institute of Human Nutrition Potsdam-Rehbrücke, Germany, and Department of Endocrinology, Diabetes and Nutrition (Chairman Prof. Dr. A.F.H. Pfeiffer), Charité-University Medicine Berlin, Campus Benjamin Franklin, Germany

Abstract: Angiogenic eye disease is among the most common causes of blindness worldwide. Current treatment approaches are insufficiently effective and partially associated with significant adverse effects. From an investigational view, the eye provides an ideal setting to observe real-time and serial observations of angiogenesis *in vivo* in humans. The current understanding of molecular biology involved in angiogenesis has already led to the identification of a number of potential therapeutic targets, some of them being highly effective angiostatic molecules. Most experimental approaches currently favour or even require the systemic administ-ration of the investigated substances (somatostatin analogues, PKC-inhibitors). However, the systemic administration of bioactive substances always risks significant systemic adverse effects. Due to the morphological characteristics of the eye, local therapies including intraocular injection or even local gene transfer might be feasible. They might provide a valuable opportunity of targeted and sustained delivery of therapeutic proteins to the retina. This review aims to outline the current understanding of the pathogenesis of proliferative diabetic retinopathy and will focus on some as yet experimental, but potentially effective new therapeutic possibilities of this disease.

Key Words: Diabetic retinopathy, hyperglycaemia, angiogenesis, neovascularisation, growth factor, anti-angiogenic therapy.

1. INTRODUCTION

Angiogenic eye disease such as proliferative diabetic retinopathy and senile macular degeneration are among the most common causes of blindness in industrialised nations. Major pathogenic characteristics are reduced perfusion of retinal vessels, increased permeability of the existing vasculature and leakage of serum substances into the eye and finally intraocular proliferation of new, but defective vessels. Until now, therapeutic options are limited. With respect to diabetic retinopathy, the control of blood glucose levels and hypertension are of outstanding importance. The introduction of photocoagulation revolutionised the therapy of angiogenic eye disease and is able to stop or at least delay the progress of disease. However, in a considerable number of cases, angiogenic eye disease tends to progress despite appropriate application of standard therapies, even including surgical therapy such as vitrectomy. In addition, photocoagulation and vitrectomy are sometimes limited by considerable side effects. Thus, further therapeutic options are desirable. Based on the improved understanding of angiogenic processes, new therapeutic options for angiogenic eye disease are underway, which might ultimately be practically useful, at least in specific clinical situations. This review will focus on presenting some of these non-classical therapeutic options and will try to evaluate their reasoning behind and their success in existing clinical studies.

2. EPIDEMIOLOGY AND CLINICAL FEATURES

Proliferative diabetic retinopathy is together with age-related macula degeneration (AMD) the major cause of blindness in industrial nations [1]. Severe forms of both diseases are characterised by retinal neovascularisation.

With respect to epidemiological data, it is important to differ between patients with type 1 diabetes (former IDDM) and patients with type 2 diabetes mellitus (former NIDDM). Clearly, there is a higher risk of developing more often and more severe ocular complications in patients with type 1 diabetes [2]. About 25% of the patients with type 1 diabetes mellitus (T1DM) develop retinopathy after 5 years and about 95% have diabetic retinopathy after 15 to 20 years after onset of diabetes. After 20 years more than 50% of patients with T1DM have suffered from proliferative diabetic retinopathy [3]. However, as the prevalence of T1DM is rather low in the general population, these cases do not account for most patients with angiogenic eye disease in the population.

In contrast, the risk of developing retinopathy is lower in patients with T2DM. About 30% of individuals with T2DM develop proliferative diabetic retinopathy after 20 years of diabetes [3]. However, due to the very high number of individuals who are not aware of their disease for a relatively long time period, about 40% of individuals with type 2 diabetes have retinal vascular damage at time of diagnosis. Despite this lower incidence of retinopathy in patients with T2DM compared to those with T1DM, T2DM accounts for the vast majority of patients with angiogenic eye disease in the United States, as the prevalence of T2DM is high with an estimated 16 million people having T2DM in the United States. Taken together, blindness is about 25 times more common in individuals with diabetes compared to those without. About 5000 new cases of blindness occur each year in the United States as a result of diabetic retinopathy [1]. The socio-economic impact is dramatic. Some studies estimated that appropriate treatment of type 1 diabetes results in savings of about $101 million and 47,374 person-years of

*Address correspondence to this author at the Department of Clinical Nutrition, German Institute of Human Nutrition Potsdam-Rehbrücke, Arthur-Scheunert-Allee 114-116, 14558 Nuthetal, Germany; Tel.: +49-33200-88-771; Fax.: +49-33200-88-777, E-mail: spranger@mail.dife.de or joachim.spranger@charite.de

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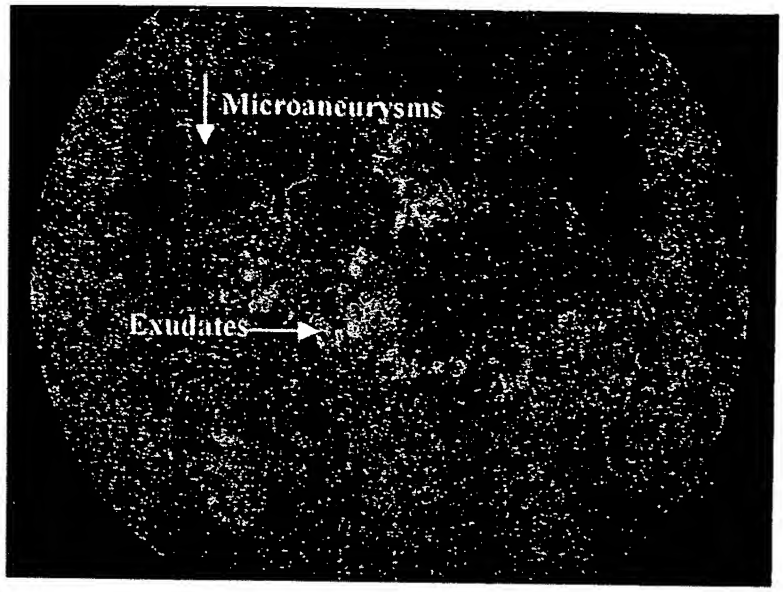

Fig. (1). Stereoscopic colour fundus photograph of non-proliferative retinopathy. The lipid deposits (exudates) and microaneurysms are characteristic retinopathic symptoms that result from damaged capillary membranes in the retina. (Courtesy from H. Heimann, Department of Ophthalmology, Charité Berlin).

sight annually and savings of $247.9 million and 53,986 person-years of sight for patients with type 2 diabetes at current treatment level [4,5].

Generally the two forms of retinopathy considered most important are the background, or non-proliferative retinopathy, shown in Fig. (1) and Fig. (2), and the proliferative form, as it is demonstrated in Fig. (3), which are simply different phases of symptomatic development. The former of these characterises the earliest stage of development and is identified by weakness and leakage of capillaries, which form small sack-shaped expansions, termed microaneurysms. The first contributing factor to pathogenesis of diabetic retinopathy is increased vascular permeability. Plasma from leaking vessels entering the extracellular space provides adhesion material for migrating leucocytes and endothelial cells. As a result reduced perfusion leads to progressive ischaemia, or disturbed blood flow, which in turn leads to hypoxia.

If intraretinal closed vessels can no longer compensate for the increasing hypoxia, vessel proliferation will be triggered

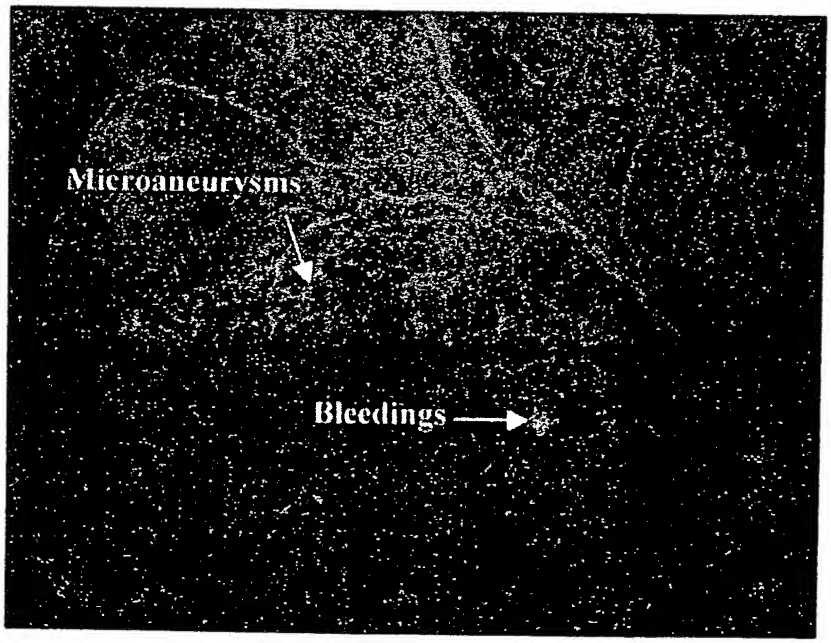

Fig. (2). The same retina as illustrated in Fig. (1), demonstrating typical non-proliferative retinopathic symptoms, as revealed by fluorescence angiography. Numerous, previously invisible, microaneurysms and bleedings are made apparent (Courtesy from H. Heimann, Department of Ophthalmology, Charité Berlin).

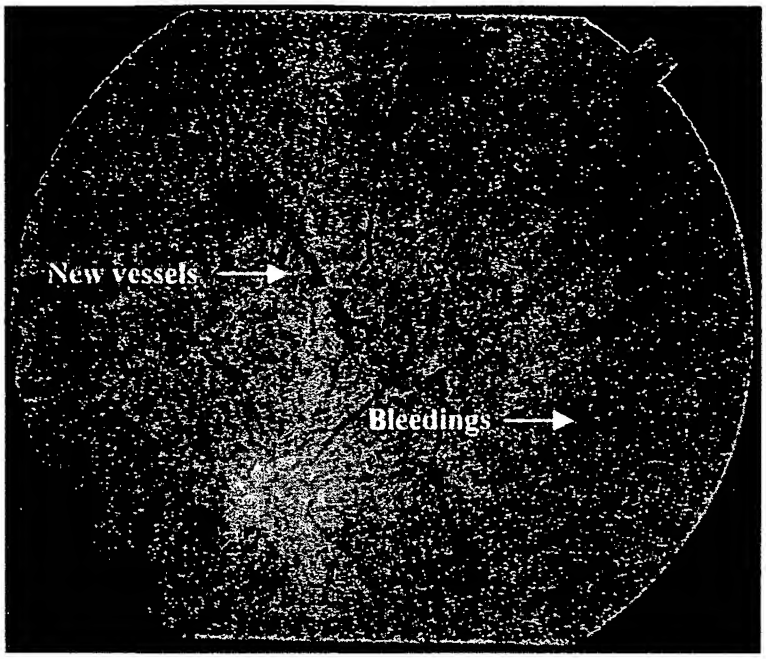

Fig. (3). Proliferative retinopathy is characterised by the development of entirely new vessels. These are physically weak and therefore rupture easily, leading to haemorrhaging (Courtesy from H. Heimann, Department of Ophthalmology, Charité Berlin).

by the release of endothelial cell stimulating growth factors, which induce angiogenesis. Because of this new vessel development, or neovascularisation, from the retina to other avascular ocular regions, for example along the surface of the vitreous gel, the non-proliferative form of retinopathy passes into the proliferative. These new vessels are very delicate and haemorrhage easily, which causes blood leakage into the eye, and therefore severe vision loss.

The pathogenesis of AMD is poorly understood. The early stages are characterised by the presence of drusen within the basement membrane of retinal pigment epithelium [6]. There are two forms of AMD. The dry form accounts for about 85% of cases. Although only 15 % suffer from the wet AMD, this form accounts for 90% of all severe vision loss from the disease and involves choroidal neovascularisation (CNV) [6,7]. It is supposed that CNV results from hypoxia of overlying retinal pigment epithelial cells, leading to the expression of angiogenic cytokines [8]. Finally, the growth of abnormal blood vessels is responsible for macula destruction, the retinal region of central vision [7]. The current treatment comprises photocoagulation and photodynamic therapy (PDT).

3. PATHOGENESIS

3.1. Biochemical Pathways Linked to Hyperglycaemia

In both type 1 and type 2 diabetes chronic hyperglycaemia and hypertension are the risk factors for the development of microvascular complications in the retina. At the moment five hypotheses about the link between hyperglycaemia and diabetic microvascular complications exist. Like it is illustrated in Fig. (4), they are the increased polyol pathway flux, the increase in advanced glycation end products (AGEs), the enhanced activation of protein kinase C (PKC), an increased hexosamine pathway flux, and at last, the reactive oxygen intermediate hypothesis.

Under the condition of permanent hyperglycaemia, glucose is reduced to sorbitol by the enzyme aldose reductase and the cofactor $NADP^+$, resulting in elevated sorbitol levels. Subsequently sorbitol is oxidised to fructose by the enzyme sorbitol dehydrogenase with concomitant reduction of NAD^+ to NADH. It is hypothesised that this pathway elevates sorbitol-induced osmotic stress, decreases the ATPase activity and cytosolic NADPH and increases the ratio of NADH to NAD^+ [9,10]. These changes may result in osmotic vascular damage, decrease of nitric oxide in endothelial cells and alterations in the cellular redox balance, as well as alterations in enzymatic activities, contributing to microvascular complications [11]. Furthermore, it was suggested that an increase in the ratio of NADH to NAD^+ stimulates the de-novo synthesis of diacylglycerol (DAG) from glycolytic intermediates, thus activating PKC-β and platelet derived growth factor-beta (PDGF-ß) receptor cascade [12]. At last, the polyol pathway might contribute to the increased AGEs in diabetic tissues [13].

The second well-characterised pathway, which is proposed to link hyperglycaemia and microvascular complications, might be that of increased AGEs. Thereby glucose or other reducing sugars react non-enzymatically with amino groups in proteins, lipids and nucleic acids to form Schiff base adducts. Subsequently these adducts undergo further reactions such as rearrangement to form Amadori products to become more stable glycation products [14]. Binding of AGE to its receptor RAGE may lead to the production of reactive oxygen species (ROS) and the activation of the mitogen-activated protein (MAP) kinase cascade with the subsequent activation of the transcription factor NF-κB, thus changing gene expression of several growth factors and cytokines like tumour necrosis factor (TNF)-α and endothelin-1 [9,11,15]. Furthermore, AGEs are able to induce intramolecular crosslinking of type I collagen, type IV collagen, laminin and fibronectin, resulting in an

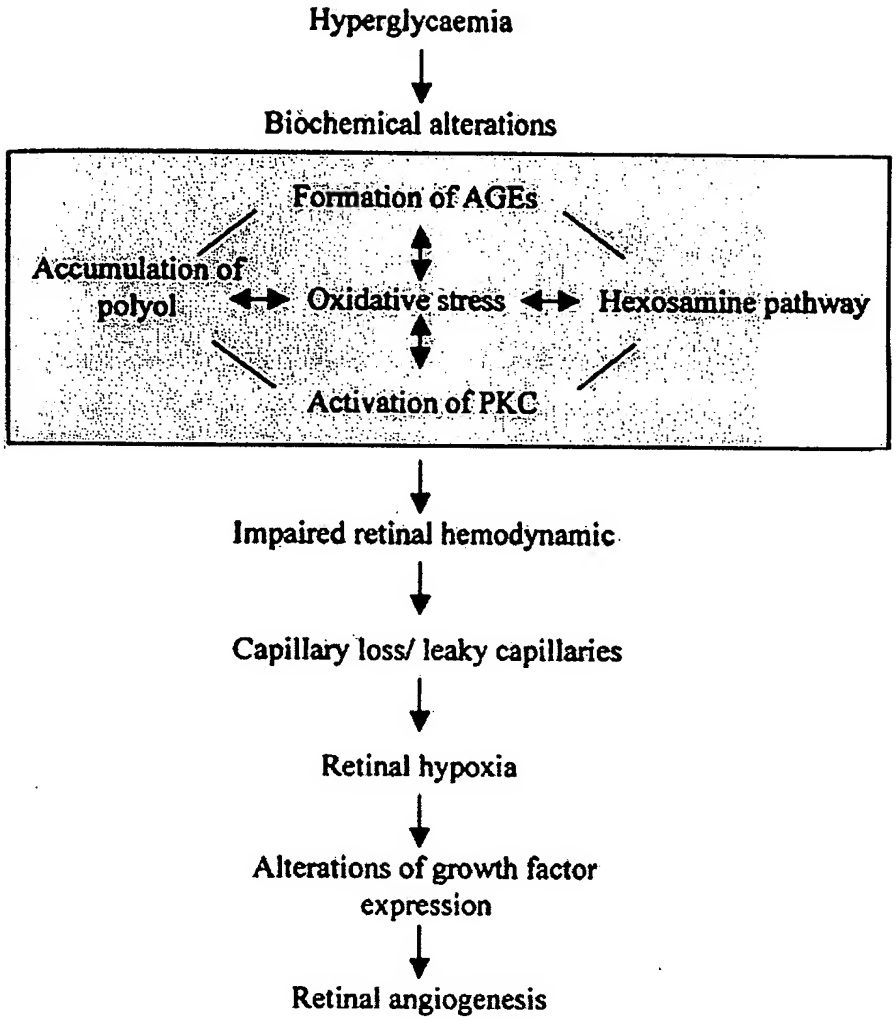

Fig. (4). Pathogenesis of retinal angiogenesis.

altered function of intact blood vessels, which includes changes of permeability and elasticity [15,16].

The third mechanism mediated by intracellular hyperglycaemia is the enhanced activation of PKC by de-novo synthesis of the lipid second messenger DAG [17-20]. Oxidative stress is also associated with PKC activation [21]. There is evidence that PKC β is particularly involved in the pathogenesis of diabetic vascular complications. King and co-workers were the first who examined the tissue-dependent PKC responses to hyperglycaemia [22]. The investigators found the beta-isoform 2 to be predominantly activated in macrovascular tissue and in the retina [22,23]. Further studies confirmed the implication of PKC β2 in the pathogenesis of diabetic retinopathy [24,25]. In both cell culture experiments and animal studies it has been shown that the activation of the PKC β isoforms mediates retinal blood flow abnormalities [26-28], nitric oxide (NO) dysregulation [29], increased synthesis of growth factors like vascular endothelial growth factor (VEGF) [30,31] and transforming growth factor (TGF) β [32], as well as the altered expression of endothelium-derived vaso-active factors such as endothelin-1 [18,33-36]. Recently, a strong interrelationship between hyperglycaemia-induced elevation of retinal oxidative stress, NO levels and the activation of PKC was suggested since an inhibitor of one single abnormality had multiple beneficial effects on the other biochemical abnormalities [37]. Inhibition of PKC β may be a promising therapeutic approach for diabetic retinopathy.

Increased hexosamine pathway flux may also contribute to hyperglycaemia-induced vascular complications. The excess of glucose leads to increased levels of fructose-6-phosphate. This intermediate enters the hexosamine pathway, but not the glycolysis pathway, providing substrates for reactions that require UDP-N-acetylglucosamine such as O-glycosylation of Sp-1. As a consequence, the transcription of factors such as plasminogen activator inhibitor 1 (PAI-1) and TGF-β1 may be increased [9]. It is hypothesised that the increased hexosamine synthesis and the O-glycosylation of Sp-1 are consequences of hyperglycaemia-induced mitochondrial superoxide overproduction [38].

The elevation of oxygen species mediated by hyperglycaemia is one of the oldest theories in diabetic complications [11]. It is suggested that oxidative and nitrative modifications in the retina occur early in the course

of development of retinopathy, as it was shown in rats [39]. All aforementioned pathways in hyperglycaemia effect each other in case of the formation of ROS [11]. So the aldose reductase pathway could decrease NADP(H) levels, thus inhibiting the reduction of oxidised glutathione. In addition, some AGEs are oxidants themselves, and AGEs can increase production of oxidants when binding to their receptors. The activation of PKC may result at least in the generation of oxidants and AGEs [11]. Again, the ROS themselves are able to activate aldose reductase and PKC, induce formation of AGEs and activate NF-κB pathway [40]. The goal of the glucose breakdown is the gain of energy by the synthesis of adenosine triphosphate (ATP) *via* oxidative phosphorylation in the mitochondria. This process is accompanied with the generation of free radicals like superoxide anion. At last, the overproduction of superoxide by the mitochondrial electron-transport chain is thought to be the linking element between the four mechanisms mentioned above [9,38,40]. The hypothesis suggests an excess of superoxide, which partially inhibits the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), increases the upstream metabolites from glycolysis, redirecting them to the four alternative pathways that cause hyperglycaemic damage [9,41]. Recently, Brownlee and co-workers demonstrated that the lipid-soluble thiamine derivative benfotiamine blocks the hexosamine pathway, the AGE pathway, the PKC pathway and the NF-κB pathway in endothelial cells and retinas from rats by increasing the activity of transketolase, the rate-limiting enzyme of the pentose phosphate pathway [42].

3.2. Alteration of Growth Factor Expression

In the healthy eye the occurrence of angiogenesis is under tight regulation of both activator and inhibitor molecules. Vascular blood supply is essential in the retina, but tissues such as the cornea and vitreous do not require a vascular system and are therefore free of vessels. This avascularity implies an existence of anti-angiogenic factors. Normally the inhibitors predominate, thereby preventing growth. Should a need for new blood vessels arise, angiogenesis activators increase in number and inhibitors decrease. This changed equilibrium prompts the growth and division of vascular endothelial cells and ultimately, the formation of new blood vessels. This change in the expression of growth regulators can lead to the proliferative diabetic retinopathy.

Numerous pro-angiogenic growth factors, such as vascular endothelial growth factor (VEGF), growth hormone, insulin-like growth factor and basic fibroblast growth factor are well documented as playing important roles in neovascularisation. The role of angiostatic molecules in retinal neovascularisation is less clear.

The most prominent growth factors VEGF, IGF-1 as well as the angiostatic protein pigment epithelium-derived factor (PEDF) and their role in diabetic retinopathy are briefly described below.

VEGF is an endothelial-specific mitogen as well as an angiogenic cytokine. It is thought to be the main stimulator of angiogenesis [43] and is characterised by its growth-promoting ability as well as its potent permeability-inducing effects [44]. In the presence of VEGF, endothelial cells are stimulated to degrade their basement membrane and to migrate with concurrent release of matrix metalloproteases (MMPs) and expression of integrins. Additionally, VEGF is capable of stimulating endothelial cells to proliferate and to survive [45]. Hypoxia is an important regulator of angiogenesis. Numerous studies have revealed the induction of VEGF expression under hypoxic conditions [4547]. This process is mediated by up-regulation of hypoxia-inducible factor (HIF)-1α, which binds to the VEGF promotor and initiates its transcription [45]. Because VEGF is a diffusible and is an oxygen regulated angiogenic factor it has been concluded that VEGF is a potential mediator of intraocular neovascularisation [48]. It has been shown that VEGF is secreted by retinal pigment epithelium (RPE) cells *in vitro* [49]. Accordingly, increased levels of VEGF were found in the vitreous and the retina of patients and laboratory animals with ischaemic retinopathies [47]. Confirmation of its critical role in retinal neovascularisation was given by the ability of VEGF antagonists to suppress the growth of blood vessels in animal models [8,50-53]. Some VEGF antagonists are currently undergoing clinical studies to test its therapeutic benefits in proliferative eye diseases. Advances in understanding of the signalling pathway of VEGF, like synergistic induction of VEGF production by activated PKC and hypoxia, resulted in concomitant investigation of drugs that can block PKC-β or MAP kinase pathway.

IGF-1 was shown to act directly as an angiogenic factor. Furthermore it was proposed to influence VEGF gene expression [54,55], thus acting as a permissive factor for maximum VEGF stimulation in angiogenesis [56]. Only a small amount of IGF-1 is circulating in the active free form, the greater part is bound to one of the six IGF binding proteins (IGFBPs), which are, among others, capable of modulating the bioavailability of IGF-1 at target cells [57]. It has been suggested that growth hormone (GH) and IGF-1 play a key role in the progression of diabetic retinopathy, after Poulsen observed regression of diabetic retinopathy (DR) after postpartal infarction of the pituitary in Sheehan's syndrome [58]. Subsequently, hypophysectomy appeared as an effective therapy of proliferative diabetic retinopathy (PDR), as it was shown by Poulsen (1966), Deckert (1967) and Sharp (1987) [59-61]. Similarly, diabetic dwarfs with low IGF-I serum levels due to a GH deficiency showed a reduced incidence of PDR compared to diabetic patients [62,63]. In addition, patients with several genetic defects of the GH/IGF-I axis had less retinal vascularisation compared to normal controls, implicating an important role of the GH and IGF-I system in human retinal vascularisation [64].

However, in the recent years some doubts about the strong association between IGF-I and proliferative retinopathy arose. Several studies revealed only a weak or rather no association between IGF-I and diabetic retinopathy. For example no relationship was found between diabetic retinopathy and plasma IGF-I levels [65], serum IGF-I levels [66-71] or vitreous free IGF-I levels (in this case after adjusting for total intravitreous protein concentration) [72]. Nevertheless, in states of elevated IGF-I levels such as pregnancy and puberty, a progression of retinopathy was demonstrated [73-75]. In addition, there are numerous studies indicating a correlation between diabetic retinopathy and IGF-I levels. A relationship was found between diabetic retinopathy and serum IGF-I concentrations [76-80] as well as vitreal IGF-I levels [81-83], even after adjusting by vitreal proteins [84]. Thereby, retinal ischaemia might be a

powerful stimulus for changes in IGF-I levels [82]. Furthermore, it was suggested that intraocular levels of IGF-I might be elevated due to a breakdown of the blood-retinal barrier [85]. However, besides the influence of IGF-I serum levels on its vitreal concentrations, an intraocular synthesis of IGF-I as well as an increased bioavailability of vitreal IGF-I, due to an imbalance between vitreal IGF-I and IGFBPs, was hypothesised by Burgos and co-workers [84].

A rapidly improved glycaemic control is frequently associated with worsening of diabetic retinopathy, probably due to the initial elevation of IGF-I levels [79,86,87]. This rapid progression of diabetic retinopathy occurs immediately after improving metabolic control and was reported to be temporary [87], in other studies a continuous progression was observed [88,89]. This phenomenon may be of clinical relevance. Thus the inhibition of IGF-I with somatostatin analogues appears to be a promising therapeutic approach for patients with PDR and improvement of metabolic control. In addition, a combined treatment with IGF-I/GH inhibitors and photocoagulation might be a successful therapy for PDR, since it was shown that after retinal scatter photocoagulation, VEGF levels were dramatically reduced, whereas the levels of IGF-I were not changed [90]. In clear contrast some authors postulate a benefit of IGF-I supplementation instead of its suppression in diabetic patients [91]. Exemplarily it was reported that co-therapy with IGF-I and insulin for 12 weeks is a more efficient therapy for the improvement of glycaemic control in type 1 diabetes than insulin treatment alone [92]. However, 8% of the patients had a significant worsening of retinopathy despite the short period of therapy. Thrailkill and associates suggested the progression of retinopathy to be dose-related, because most of these patients received the two highest doses of IGF-I [92]. There are great concerns about IGF-I related ophthalmologic changes after all. The worsening of retinopathy might be also associated with long-term therapy at the lowest IGF-I doses, so the safety of co-therapy with IGF-I and insulin has to be carefully investigated before using it as new treatment in patients with type 1 diabetes [93].

Several naturally occurring inhibitors of angiogenesis have been identified, including PEDF, endostatin and angiostatin. PEDF is the most potent inhibitor of angiogenesis in the eye. Besides its anti-angiogenic effects, PEDF was shown to be neuroprotective and might be involved in neuronal differentiation in the retina [9498]. Tombran-Tink and associates were the first who isolated PEDF from medium conditioned by human fetal RPE cells [99]. PEDF acts *via* induction of apoptosis in endothelial cells that are forming new vessels [100,101], whereas the neuronal survival might be promoted through PEDF-induced activation of NF-κB [102]. Its production by retinal cells is positively correlated with oxygen concentrations, implicating its loss in ischaemia-driven neovascularisation in the retina [103]. Gao and colleagues demonstrated an increased VEGF/PEDF ratio in the retina of rats with ischaemia-induced neovascularisation, and thus suggesting that the imbalance between angiogenic stimulators and inhibitors may contribute the development of retinopathy [104,105]. In animal models, systemically or intravitreal administered PEDF was able to block neovascularisation in ischaemia-induced angiogenesis [106,107]. Additionally, it was demonstrated that vitreous and retinal levels of PEDF are decreased in diabetic patients with proliferative diabetic retinopathy [108-110]. Interestingly, Boehm and co-workers demonstrated that VEGF levels in the aqueous humour are increased in both diabetic patients with and without proliferative retinopathy compared to non-diabetic controls, whereas PEDF levels are only decreased in diabetic patients with proliferative retinopathy [111]. The same group found that the endogenous content of PEDF in aqueous humour predicted progression of diabetic retinopathy, whereas VEGF content had no predictive quality [112]. Because of its efficient anti-angiogenic properties, PEDF has been proposed to be successful in the therapy of retinopathy, either delivered systemically, locally or in gene therapy.

4. THERAPIES FOR DIABETIC RETINOPATHY

4.1. Standard Therapy

It is now well-established that hyperglycaemia and hypertension are major contributors to the development of diabetic microvascular complications. The large clinical trials The Diabetes Control and Complications Trial (DCCT) [113] and The UK Prospective Diabetes Study (UKPDS) [114] provided evidence that an intensive metabolic control prevents microvascular complications in patients with type 1 and 2 diabetes. In patients with type 2 diabetes, blood pressure control was shown to effectively prevent diabetic retinopathy. The current treatment of proliferative retinopathy is the retinal photocoagulation. This method can maintain vision, but not improve it. Laser treatment can prevent blindness in at least one eye in 80-90% of cases and is therefore highly effective. However, photocoagulation could be followed by several complications [115]. These side effects of laser treatment include diminished night vision, pain and loss of precise vision [116] as well as constriction of peripheral visual field [116, 117], changes in colour vision [118] and damage of the underlying neural retina [119]. Furthermore, in some patients the preventive measures and photocoagulation fail to prevent blindness. It follows that there is a need for new non-invasive therapies with fewer side effects, which are also effective in the treatment of early stages of retinopathy. To date, various new approaches in the therapy of retinopathy were developed. Because it is known that several growth factors contribute to the development of retinopathy, the majority of new therapies consists of the systemic or local supply of growth factor antagonists or the blockade of signalling pathways, as well as genetic approaches in order to inhibit the growth of abnormal blood vessels in the course of angiogenic eye disorders.

4.2. Alternative Therapeutic Approaches

4.2.1. Systemic Drug Administration

4.2.1.1. Aldose Reductase Inhibitors

An approach to the therapy of diabetic complications is the inhibition of the polyol pathway through inhibition of its first enzyme aldose reductase. To date several aldose reductase inhibitors (ARIs) have been tested in clinical trials, including sorbinil and tolrestat.

In the Sorbinil Retinopathy Trial (SRT) the worsening of retinopathy was not significantly different in the sorbinil-

and in the control group [120]. The observed slower progression rate in the microaneurysm count among patients receiving sorbinil is a finding of uncertain clinical importance. Additionally, about 7% of the patients assigned sorbinil developed hypersensitivity reaction in the first three months [http://www.nei.nih.gov/neitrials/ index.html, last updated 1999-10-23]. Therefore, the drug was withdrawn from the market. Similarly, tolrestat was withdrawn in 1996, principally due to its low efficacy [10]. Presently, only epalrestat is still available on the market [10]. In cell culture experiments it was shown that epalrestat could inhibit glucose-induced PKC activation as successful as the PKC inhibitor LY333531, additionally the drug decreased proliferation activities, the expression of PDGF-β receptor, and normalised accelerated oxidative stress in smooth muscle cells [12]. In addition, epalrestat was shown to inhibit increased expression of endothelial adhesion molecules such as intercellular adhesion molecule-1, possibly through inhibition of a PKC-dependent pathway or increased endothelial NO production [121]. Furthermore, it was observed that treatment of diabetic patients with epalrestat for two months lowered the N-ε-carboxymethylated lysine (CML) levels in erythrocytes, one of the most frequently measured and abundant AGEs in tissues [13]. There was a significant correlation between CML and polyol pathway metabolites, suggesting a linkage between the polyol pathway and the formation of AGEs. However, in the clinical trial conducted by Asano and colleagues, the treatment of diabetic patients with epalrestat resulted in only minimal reduction of sorbitol accumulation in erythrocytes, whereas another ARI, namely fidarestat, was more effective [122]. Similar results were obtained by Sobajima and co-workers [123]. At this time, the preventive effects of ARIs are predominantly examined on diabetic neuropathy, and, to the lesser extent, on retinopathy [122-125]. In conclusion, several aldose reductase inhibitors have been developed but withdrawn because of side effects and lack of improvement of diabetic complications. However, the research for new drugs in molecular modelling studies is still continuing and aiming to discover compounds that show sufficient *in vivo* activity as well as a good selectivity, thus reducing toxic side effects [10].

4.2.1.2. Inhibitors of Protein Glycation, Especially Aminoguanidine

In laboratory models, aminoguanidine was shown to have beneficial effects on several diabetes-induced alterations of tissue function and structure [126]. In the retina, aminoguanidine could inhibit some lesions in diabetic rats, such as acellular capillaries and microaneurysms [127,128]. In addition, it decreased NO levels in the retinas from diabetic rats [129]. In contrast, a study conducted by Azal and co-workers failed to show preventive effects of aminoguanidine on retinal vasculature in rats [130]. In diabetic dogs aminoguanidine significantly inhibited the development of retinopathy, but without reduction of systemic AGEs such as Hb-AGE and pentosidine [131]. Finally, the mechanism by which aminoguanidine exerts its beneficial effects in diabetic complications is not clear [128]. Since it is known that aminoguanidine does not simply inhibit the formation of AGEs, but also acts as an inhibitor of inducible nitric oxide synthase and oxidants, the positive effects of this agent may be due to mechanisms other than the inhibition of AGE pathway [11,132]. Its action in retinopathy might be mediated in part by its antioxidant properties, by inhibition of NO production [129] and lipid peroxidation [133]. Due to the presence of limiting toxicity, clinical trials using aminoguanidine have been inconclusive [11]. The hydrazine structure of aminoguanidine might present a severe limitation for a safe treatment without interfering with other biochemical reactions in the body, therefore it will be very difficult to design selective and efficient inhibitors of AGE [134]. Other drugs with the potential of blocking protein glycation and AGE formation are currently examined in laboratory models, such as pyridoxamine, which was shown to inhibit the formation of AGEs from Amadori compounds as well as the formation of advanced lipoxidation end products [135-137], pyridoxamine analogues [138] and thiamine [139].

4.2.1.3. Antioxidants

Vitamin E is one of the best studied antioxidants and was proposed to be involved in the activation of DAG-PKC cascade [24]. Several studies have shown that vitamin E could ameliorate the accumulation of DAG through activating DAG kinase, which metabolises DAG to phosphatic acid [32,140,141], as well as prevents glucose-induced PKC activation [23,142,143]. In addition, vitamin E could decrease leukostasis in diabetic rats [28]. In contrast, alpha-tocopherol-deficient rats did not develop characteristic changes of retinopathy, with the exception of moderate retinal capillary basement membrane thickening [144]. Furthermore, vitamin E had no effect on the development of acellular capillaries in the retina of diabetic rats [136]. Supplementation with both vitamin C and E as well as a multi-antioxidant mixture inhibited the development of acellular capillaries and the number of pericyte ghosts in the retinas of diabetic rats, whereas the thickening of basement membranes was not effected [145].

Some clinical studies were conducted to investigate the role of antioxidants in proliferative eye diseases. Bursell and associates examined the impact of vitamin E on the retinal blood flow in type 1 diabetic patients in an 8-month randomised double-masked placebo-controlled crossover trial [146]. The high dose of 1.800 IU/day vitamin E increased the blood flow significantly in diabetic patients with 88% normalisation compared to non-diabetic patients. Additionally, vitamin E reduced the PAI-1 levels in diabetic patients. However, this trial was only short-termed, therefore a potential development of retinopathy could not be observed. It was the only clinical trial where positive effects of vitamin E on alterations in the course of retinopathy could be shown. Apart from that, other studies made the investigation of age-related macular degeneration (AMD) a subject of discussion, but not retinopathy. Exemplarily, in the study conducted by Mares-Perlman and associates a beneficial role of vitamin E for AMD remained doubtful [147]. The Physicians' Health Study I showed a slight but not significant reduction in the risk of AMD after supplementation of either multivitamins or vitamin E [148,149]. Therefore the Physicians' Health Study II will provide the first randomised trial data of antioxidant vitamin research, testing beta-carotene, vitamin C and E as well as multivitamins [149]. Taylor and co-workers did not find any preventive effect of 500 IU/day vitamin E in the development

or progression of early or later stages of age related macular degeneration in a four year study with older healthy volunteers [150]. Currently, the effect of the treatment with high dose vitamin E (1600 IU) prior to and following laser photocoagulation is examined in diabetic patients with macular oedema in phase I clinical trial.

In conclusion, the positive effect of vitamin E or other vitamins on the development of retinopathy remains doubtful. In animal studies, contradictory results were obtained, and the clinical trial conducted by Bursell and associates might be interpreted as a request to confirm these results in a larger long-term trial with several endpoints. In some cases, the combination of multivitamins and minerals indicated modest reduction of AMD, but mainly conflicting results were obtained. In general, antioxidants such as vitamin E may not play a key role in protecting against proliferative eye diseases like retinopathy and AMD.

4.2.1.4. Inhibitors of PKC, Especially LY333531

Chronic hyperglycaemia increases cellular levels of DAG, which can activate PKC in endothelial cells, especially the beta isoforms. PKC β, an ATP dependent kinase, is one of the 13 known isoforms of the PKC family, and seems to play an important role in the process of angiogenesis [19]. The activation of PKC could have several effects on vasculature, including changes in smooth muscle contractility, decreases in retinal blood flow, increases in basement membrane protein synthesis, endothelial permeability and stimulation of angiogenesis [19,24]. PKC activation may also result in the alteration of cytokine levels such as endothelin-I, VEGF and transforming growth factor-beta, as well as increases in inducible NO synthase gene expression and NO production [24,151]. To block angiogenic processes several inhibitors of PKC with different affinities for the PKC isoforms were tested, among them Rottlerin, indolcarbazoles, PKC412, bisindolylmaleimides and the very promising LY333531 [24]. LY333531 is a macrocyclic bisindolylmaleimide compound and it is highly selective in inhibiting the beta isoforms. This specific PKC β inhibitor has little effect on other ATP dependent kinases such as calcium calmodulin or tyrosine kinase [26,152]. Therefore it promises to have little side effects during a systemic administration. In cell culture experiments, LY333531 was able to inhibit the glucose-induced PKC activity without lowering the DAG levels [32]. The PKC inhibitor also prevented glucose-induced release of arachidonic acid and prostaglandin E_2 in mesangial cells, thus avoiding inhibition of Na^+-K^+-ATPase activity [32]. Hyperglycaemia-induced inhibition of Na^+-K^+-ATPase due to PKC β activation changed cellular functions, thereby contributing to diabetic microvascular complications [32]. In animal studies, LY333531 could normalise elevation in retinal PKC activity induced by hyperglycaemia [27,153], attenuated the development of endothelium-dependent vasodilatation [154] and increased retinal blood flow [27]. LY333531 prevented the increased mRNA expression of endothelin-1, PDGF β [36], TGF-β1 and extracellular matrix components such as fibronectin and collagen [32]. Furthermore, the compound normalised hyperglycaemia-induced decreases of Na^+-K^+-ATPase and calcium ATPase activity in the retina [153]. Since LY333531 could successfully block angiogenesis in animals [18], it was tested in humans. Evidence of its bioavailability and of no adverse effects in patients in clinical phase I studies was given by Demolle and Aiello [19]. The administration of LY333531 for one month was shown to normalise blood flow in diabetic patients [19,155]. Beckman and colleagues examined the effect of LY333531 on endothelium-dependent vasodilatation in fifteen healthy non-diabetic humans exposed to hyperglycaemia in a randomised, double-blind, placebo-controlled cross-over study [156]. They found that the inhibition of PKC β ameliorated vascular dysfunction under hyperglycaemic conditions, achieved by a hyperglycaemic clamp for six hours [156]. Recently, preliminary results from the Protein Kinase C β Inhibitor Diabetic Retinopathy Study (PKC-DRS) were presented on the 18th Congress of the International Diabetes Federation by Milton and co-workers [157]. In this double-masked, placebo-controlled, parallel study 252 patients with moderately severe to very severe nonproliferative diabetic retinopathy randomly received placebo or one of three doses of LY333531 for up to 48 months. The authors did not find a significant effect on the progression of diabetic retinopathy, but a potential beneficial effect of LY333531 in reducing moderate visual loss was assumed. However, with respect to the development of proliferative diabetic retinopathy, the study may have been underpowered. According to such uncertainty, the submission of LY333531 for eye indications has been delayed and further studies are required. None of the clinical trials reported adverse effects associated with LY333531.

Another specific inhibitor of PKC β2, the compound LY379196, may also be a potent agent to prevent neovascularisation in the eye. Chibber and associates found that the PKC inhibitor prevented leukocyte adhesion to bovine retinal capillary endothelial cells [158]. They hypothesised that one of the underlying mechanisms could be inhibition of the PKC beta 2-dependent phosphorylation of [beta] 1, 6-acetylglucosaminyltransferase as a regulatory mechanism in mediating increased leukostasis and capillary occlusion in diabetic retinopathy. In contrast, LY379196 reduced the replication of bovine retinal pericytes and increased the percentage of resting cells, implicating a synergistic action with glucose in the course of pericyte loss [159]. Therefore the effect of PKC β inhibition should be separately evaluated in earlier and later stages of retinopathy. Another drug named CGP 41251 or PKC412, a derivate of staurosporine, is not only able to inhibit several PKC isoforms, but is also an inhibitor of phosphorylation by VEGF and PDGF [160]. In mice this orally given drug was shown to completely inhibit ischaemic retinal neovascularisation [160]. In transgenic mice over-expressing PDGF in the retina, PKC412 reduced the incidence of severe retinal detachments [161]. No adverse effects of the last mentioned PKC inhibitor were reported, despite its only partial selectivity. Phase I trials with this staurosporine derivate are ongoing [19], as well as a phase II trial of PKC412 monotherapy in patients with acute myeloid leukaemia and patients with myelodysplastic syndrome [http://www.clinicaltrials.gov, last updated 2003-July]. Recently another phase I/II trial examining the safety and efficacy of PKC412 was finished [162]. 141 patients with type 1 or 2 diabetes and nonproliferative diabetic retinopathy or mild PDR and diabetic macular oedema were randomised

to one of four treatment groups (placebo, 50, 100 or 150 mg/d PKC412, orally administered). After three months the PKC inhibitor reduced retinal thickness in the PKC412 groups in a dose- and time dependent manner. Additionally, an improvement in visual acuity was observed, but only in the 100 mg/d group the result was significant. The author concludes that the PKC inhibitor may reduce macular oedema and improve visual acuity in diabetics at doses of 100 mg/d or higher [162]. Most interestingly, the authors also observed an effect on glucose homeostasis. Thus, the direct beneficial effects on the macular oedema may be underestimated. However, various patients had elevated transaminases suggesting liver toxicity of the drug. Thus, a systemic treatment with this drug appears to be inappropriate at this stage, but local application may be possible. Minor, but more common side effects were gastrointestinal events like nausea, diarrhoea and vomiting.

4.2.1.5. Somatostatin Analogues

Somatostatin was firstly defined in the 1960s as a naturally occurring inhibitor of GH release. It is synthesised as a precursor molecule, which undergoes an enzymatic cleavage into two active peptides of 28 and 14 amino acids [163,164]. Its receptors are located ubiquitously through the body. The physiological actions of somatostatin are primarily inhibitory, like inhibition of pituitary GH secretion, suppression of GHRH secretion and TSH secretion [117]. A complete description of somatostatin action is given by Boehm and Lustig and by García de la Torre *et al.* [117,164]. The hypothesis that GH and IGF-I play a key role in the progression of proliferative retinopathy, advances a key modulator of GH action somatostatin to the use as a therapeutic drug. Therefore, somatostatin receptors are an attractive target for inhibition of neovascularisation. The family of somatostatin receptors consists of five major subtypes named SSTR1-5 [163,165]. Splice variants for the type 2 receptors (SSTR2A and SSTR2B) have also been identified [119]. Somatostatin has a very short half-life of 1.5-3 minutes. For pharmaceutical action, a continuous infusion would be required to maintain effective concentrations in the target organ. In contrast, synthetic analogues of somatostatin have longer half-lives. These analogues incorporate four amino acids in a specific configuration [117]. While natural somatostatin has the same affinity to all receptor subtypes, the synthetic analogues bind to the five receptors with different affinities. Most of the studies with the aim of preventing neovascularisation have used somatostatin analogues with very high affinity to SSTR2 and lower affinity to SSTR3 and SSTR5 [164], because it was shown that inhibition of GH release is most sensitive to receptor type 2 agonists [166] and SSTR5 may be involved in inhibiting GH release [167]. The most frequently used drug in human studies is octreotide, also named SMS 201-995 or Sandostatin (Novartis).

Several clinical trials and case reports examined the effect of synthetic somatostatin analogues on diabetic retinopathy. An overview is given in Table 1. The first case report was given by Lee and colleagues [168]. A female patient with insulin-dependent diabetes mellitus (IDDM) under glycaemic control developed haemorrhages and microaneurysms in both eyes. Subsequently she was treated with octreotide for three months, maintaining normoglycaemia. At the end of the trial, a complete resorption of haemorrhage and reduction of microaneurysms were observed. In addition, GH levels and IGF-1 levels were decreased. During the period of the trial no side effects occurred. Follow-up examination after two months without octreotide showed a worsening of the eyes. Later on, Hyer and associates examined the effect of octreotide on GH and IGF-I levels in patients with type 1 diabetes and normal controls [169]. Three infusions daily failed to suppress GH levels in both patients and controls. Continuous infusion for three days completely suppressed GH levels in control subjects, whereas treatment for up to 20 weeks resulted in only partial suppression in diabetics. The authors found that diabetics with retinopathy seemed to be more resistant to the GH-suppressing effects of octreotide than normal subjects. IGF-I levels were decreased in both groups. The researchers found no clear effect on retinopathy appearance as well as no prevention of retinal haemorrhage and further laser treatment. However, in one patient with preproliferative retinal changes, no progression was observed. In the study of Shumak and co-workers, six patients received octreotide, also resulting in only incomplete inhibition of GH [170]. The suppression of GH was greater in the patient who received continuous infusion than in patients receiving periodic injections. During octreotide therapy visual acuity was improved. However, due to the small sample size, no definite statement could be given. During the clinical trial conducted by Kirkegaard and associates, patients with IDDM (type 1 diabetes) and mild retinopathy were treated with octreotide for 12 months [171]. No difference in eye morphology could be observed between octreotide-treated group and controls. This study revealed that the treatment with octreotide for one year does not change the course of early diabetic retinopathy. McCombe and co-workers observed 17 patients with PDR for three months, eleven of them received the somatostatin-analogue somatuline *via* subcutaneous pumps, eight of them completed the study [172]. After four weeks an improvement of retinopathy was seen in two patients, whereas the six remaining patients showed neither improvement nor deterioration. In the control group three of the six patients showed some worsening of retinopathy [172]. Similarly, Mallet and colleagues examined the effect of octreotide in four type 1 diabetics with PDR that could not be treated by photocoagulation [173]. In all four patients visual acuity improved within 3-6 months and neovascularisation was stopped, even a regression was seen in two patients. No effect of octreotide on macular ischaemia could be observed. GH levels were partially decreased in the first six months, but after that resistance to octreotide began to develop. Accordingly, initially decreased circulating IGF-I levels returned to baseline after six months. Because of ethical reasons no control group was recruited. Another case report was given by Kuijpers and associates. They treated a patient with worsening of visual acuity with octreotide [174]. During the treatment visual acuity was improved and macular oedema were decreased. After stopping treatment, visual acuity worsened and macular oedema recurred. Grant and colleagues examined the effect of octreotide in 11 patients with NPDR or mild PDR as well as 11 controls [175]. They observed retardation of the development of high-risk PDR in patients receiving octreotide in combination with conventional diabetes management. During the 15 months subjects of the control group with only conventional management required photocoagulation more frequently.

However, both of the results did not achieve statistical significance. Boehm and co-workers reported about beneficial effects on the retina of patients with PDR when treated with octreotide [176]. They observed risk reduction of dense vitreous haemorrhages and vitreoretinal surgery after 12 months. Visual acuity remained stable under octreotide treatment, whereas it was significantly decreased in the control group. Additionally, in patients receiving octreotide the development of neovascularisation was decreased compared with controls. In summary, in patients with diabetic retinopathy, treatment with somatostatin analogues displayed some beneficial effects, especially in patients suffering from the high risk form of PDR. On the other hand, in early retinopathy no preventive effects on further progression of the disease could be observed. Therefore the effect of somatostatin analogues on the single stages of retinopathy has to be distinguished and further clarified.

Somatostatin analogues were shown to reduce insulin requirements in diabetic patients [171-176]. It should be mentioned that the use of somatostatin and its analogues is accompanied with several side effects. It was reported that continuous infusion of natural somatostatin could be followed by post-infusion hypersecretion of GH [117]. An interruption of octreotide treatment resulted in a subsequent increase of GH levels [173]. Furthermore, studies using somatostatin analogues reported abdominal discomfort and cramps [169,170,172,176], increased bowel movements [172,176], bloating [173], mild diarrhoea [170,173] as well as severe diarrhoea [171], constipation [173], gall bladder sludge [171,173], slight changes in thyroid hormones [171] and the trend to hypoglycaemia [169]. With these relatively rare adverse effects, the treatment with octreotide is regarded to be a safe treatment modality [117].

Another possibility to intervene in the GH/IGF-I axis is the blockade of GH receptors. There was one clinical trial examining the effect of pegvisomant, a GH receptor antagonist [177]. A subcutaneous injection of the drug for twelve weeks had no positive effects on the neovascularisation in the eye compared to controls, despite reduced serum IGF-I levels.

4.2.2. Local Therapies

The use of systemic therapies may have side effects. Due to selective permeability of the blood-retinal barrier, high

Table 1. Clinical trials Using Octreotide for the Treatment of Angiogenic Eye Disease

Author	Subjects	Intervention	Results
Lee *et al.* 1988 [168] Case report	1 patient with IDDM and progressive retinopathy	50 µg octreotide, injected 3 times daily for 3 months	Decreased GH and IGF-1 levels Complete resorption of haemorrhage and reduction of microaneurysms
Hyer *et al.* 1989 [169]	9 patients with IDDM 6 normal controls	(a) 50 µg up to 500 µg octreotide, injected subcutaneously 3 times daily for 8 to 20 weeks (b) 500 µg/day octreotide by continuous subcutaneous infusion for 3 days to 16 weeks	(a) No GH suppression, improvement of visual acuity in 3 of 4 patients (b) Reduction of GH-levels, no change in visual acuity in 5 patients, improvement in one patient, no progression of prePDR (1 patient) (a+b) Reduction of serum IGF-1 levels, no improvement of retinal haemorrhages
Shumak *et al.* 1990 [170] Case report	6 patients with IDDM and nonproliferative retinopathy	(a) 50 µg octreotide, injected subcutaneously twice daily in 3 subjects (b) 50 µg octreotide, injected subcutaneously 3 times daily in 2 subjects (c) 4 µg/h to 8 µg/day by continuous subcutaneous infusion in 1 patient	(a-c) Decrease of GH levels, Increased visual acuity In 4 subjects retinopathy levels were unchanged, in 2 subjects improved
Kirkegaard *et al.* 1990 [171]	20 patients with IDDM and mild retinopathy, 11(7) treated, 9(7) controls	starting with 50 µg octreotide and increasing to 400 µg/day by continuous subcutaneous infusion for 12 months	Serum GH and IGF-1 levels were moderatly decreased, more during daytime, but less at night In 1 patient improvement of eye morphology, unchanged in the others
McCombe *et al.* 1991 [172]	17 patients with IDDM and PDR, 11(8) treated, 6 controls	1500 µg/day Somatuline (BIM23014) by continuous infusion for 3 months	GH suppression daytime, reduction of IGF-1 levels No changes of visual acuity in either group, clinical and angiographic improvement in 2 patients, no effect in 6 patients
Mallet *et al.* 1992 [173]	4 patients with IDDM and long-term PDR	400 µg/day octreotide by continuous subcutaneous infusion for 6-20 months	Partial decrease in GH and IGF-1 levels, resistance after 6 months In 2 patients halting the PDR, in 2 others regression of PDR,improvement of visual acuity
Kuijpers *et al.* 1998 [174] Case report	1 patient with idiopathic cystoid macular oedema	100 µg octreotide 3 times daily, injected subcutaneously for 10 weeks, pause of 2 weeks, 100 µg octreotide once daily, injected subcutaneously for 3 months, second stop of 4 weeks, resumption of octreotide treatment	After 6 weeks improvement of visual acuity, than no further improvement, in each therapy pause worsening of visual acuity, decreased macular oedema during octreotide treatment with recurrence after stopping treatment
Grant *et al.* 2000 [175]	23 patients with type 1/2 diabetes and either severe NPDR or early PDR, 11 treated and 12 (11) controls	200-5000 µg/day octreotide by subcutaneous injections 4 times daily or by continuous subcutaneous infusion for 15 months	Significant suppression of serum IGF-1 values Lower progression of retinopathy with octreotide, decrease in requirement of photocoagulation
Boehm *et al.* 2001 [176]	18 patients with type 1/2 diabetes and advanced PDR, 9 treated and 9 controls	300 µg/day octreotide by subcutaneous injection for 3 years	Visual acuity remained stable Risk of vitreous haemorrhage and the need for vitreo-retinal surgery was reduced, neovascularisation was decreased

doses of a drug are required to achieve effective concentrations in the target organ [8,116]. The systemic inhibition of angiogenesis would compromise critical vascular responses to ischaemic events in patients with ischaemic heart disease and cerebrovascular and peripheral vascular disease [8]. Therefore, local therapies for ocular eye diseases were developed, e.g. repeated intra-ocular injections and ocular gene therapy.

4.2.2.1. Local Injections

Growth Factors

In a monkey model of choroidal neovascularisation, repeated intravitreal injections of an antigen-binding fragment of a recombinant humanised monoclonal antibody directed toward vascular endothelial growth factor (rhuFab VEGF) prevented formation of clinically significant CNV [178]. A current clinical trial (phase I+II) should evaluate the safety and efficacy of intravitreal injections of rhuFab V2 (also named Lucentis, ranibizumab) in combination with Verteporfin photodynamic therapy in subjects aged 50 years or older with neovascular AMD. The study started in march 2003 [http://www.clinicaltrials.gov, last updated 2003-09-04].

Another type of anti-VEGF agent, the anti-VEGF pegylated aptamer EYE001 (Macugen) was shown to inhibit VEGF-dependent angiogenesis in the cornea of rats as well as neovascularisation in a model of retinopathy of prematurity (ROP) in mice [179]. A phase IA clinical study showed that a single intravitreal injection of EYE001 in 15 patients with subfoveal CNV secondary to AMD could be administered safely up to 3 mg/eye. No significant ocular or systemic side effects were noted [179]. Subsequently, a phase II multiple injection study of anti-VEGF therapy with and without photodynamic therapy was performed [180]. Because of the lack of controls and small size of subjects, no final conclusion could be made about the therapeutic benefits regarding visual acuity. The majority of patients showed stabilised or even improved vision. Some side effects probably associated with administration of the anti-VEGF aptamer were observed, including vitreous floaters, mild anterior chamber inflammation, eye pain and increased intra-ocular pressure. Macugen is now in a fully enrolled phase III development programme for the treatment of AMD [181,7]. Another ongoing phase II clinical trial is designed to establish the safety and preliminary efficacy of intravitreal injections of EYE001, given to patients with clinically significant diabetic macular oedema. The expected total enrolment comes to 176 participants, the estimated completion date is June 2004 [http://www. clinicaltrials.gov, last updated 2003-09-04].

Extracellular Matrix Factors

An early pathological change in the course of diabetic retinopathy is the digestion of the vascular basement membrane, mediated by components of the plasminogen activator-plasmin system and members of the matrix metalloproteinases (MMP) family [182]. Therefore, the inhibition of interactions of angiogenic endothelial cells with components of extracellular matrix may be a therapeutic target to defeat pathologic angiogenesis [183]. Steroid compounds were shown to be anti-angiogenic and may up-regulate the expression of the extracellular matrix enzyme PAI-1, thus changing the extracellular matrix around the endothelial cell [118]. The action of the steroid compound triamcinolone acetonide, a known drug for arthritis, is based on its anti-inflammatory, anti-angiogenic and anti-vascular permeability effects. An open, uncontrolled study evaluated the use of intravitreal triamcinolone acetonide administered immediately after PDT [184]. Despite being small and uncontrolled, the study indicated that the adjunctive use of triamcinolone might improve visual outcomes. The steroid compound might also be effective in reducing the central macular thickness and improving visual acuity in patients with macular oedema [185]. However, Benhamou and associates demonstrated that the improvement of visual acuity after intravitreal injection of triamcinolone acetonide was only transient in nature [186]. Even patients receiving repeated injections lost vision after initial positive response. Additionally, in patients with proliferative retinopathy who underwent vitrectomy, the subsequent injection of triamcinolone acetonide showed no benefit regarding post-operative complications [187]. Fluocinolone acetonide is an investigational new drug currently under examination for the treatment of subfoveal CNV and diabetic macular oedema in the Macular Degeneration Study (CDS-FL-003). An implant in the patient's eye should slowly release fluocinolone over 3 years. However, there are still concerns about elevated intraocular pressure and progression of cataracts after treatment with triamcinolone and fluocinolone. Anecortave acetate is the third angiostatic steroid compound under examination at present. The drug was shown to inhibit tumour growth in mice [188] as well as neovascularisation in a mouse ROP model [189]. Alcon Research, Ltd. is conducting two Phase II studies in the US and Europe for 12 months to assess the safety and efficacy of anecortave acetate for the treatment of AMD as a single therapy or with photodynamic therapy. D'Amico and colleagues reported preliminary results from its phase II clinical trials after six months, demonstrating a trend of vision preservation and lesion inhibition in patients with exudative age-related macular degeneration after administration of 15 mg anecortave acetate, but the results did not achieve statistical significance [190]. Using the drug for treatment of sub-foveal CNV, visual acuity was significantly better than in controls, and lesion growth inhibition was increased [191]. Currently a phase III trial is ongoing to investigate the long term efficacy and safety of posterior juxtascleral injections of anecortave acetate 15 mg in patients with AMD [http://www.clinicaltrials.gov, last updated 2003-09-04].

4.2.2.2. Ocular Gene Therapy

The systemic administration of bioactive substances always risks significant systemic adverse effects, and the blood-retinal barrier has to be defeated. In contrast, the local administration of anti-angiogenic agents into the eye would avoid systemic adverse effects. However, in order to maintain effective levels of these angiostatic proteins with short half-lives in the eye, frequently repeated intraocular injections would be necessary, possibly resulting in local complications like intraocular infection, vitreous haemorrhage and retinal detachment [8]. To avoid these problems, research in the field of ocular gene transfer has been progressed in the recent years. Several vectors are used for the delivery of therapeutic genes to appropriate cell targets in a single procedure. A systemic delivery fails to deliver the genes to

ocular structures; therefore a surgical procedure by means of intravitreal or subretinal injections is necessary to achieve gene transfer [192].

The eye offers ideal conditions for the development of gene therapy. It is separated from the systemic circulation, highly compartmentalised, readily accessible, of small size, immune privileged and optically transparent. Therefore the accurate delivery of vector suspensions is possible, only tiny volumes of vector suspension are required. Additionally, microsurgical interventions could be carried out under direct visualisation, and therapeutic effects may be immediately observed and evaluated [8,193,194]. The immune-privileged status of the eye is partially due to sequestration of antigens from systemic circulation as well as to the suppression of delayed-type hypersensitivity, preventing ocular tissues from cytotoxic T cells and complement-fixing antibodies [194]. Furthermore, several immunosuppressive factors could be found in the eye. In gene therapy this immune privilege may give the benefit of successful and long-term transgene expression at appropriate levels [192,194].

Recombinant viruses have been used most successfully in this area, whereas gene expression by non-viral vectors is generally inefficient and short-lived compared to viral vectors [192,195]. Viral vector models evaluated for successful gene delivery to ocular cells are recombinant adenoviral vectors (Ad), recombinant adeno-associated virus (rAAV), lentiviral vectors and retroviral vectors.

Recombinant adenoviral vectors can efficiently transduce RPE cells [196]. These vectors have a high gene transfer efficiency, but they were shown to be immunoresponsive, resulting in only short-term transfection [8,192,197]. Furthermore, the virus remains as episomal DNA within the cell, but is not incorporated into the host genome, which is responsible for the transient nature of adenoviral vector gene expression [196].

Recombinant adeno-associates viruses can transduce a wide variety of tissues, including various cell types in the retina like RPE cells and photoreceptor cells [196,198], and have shown very little toxicity [199]. Additionally, gene transduction mediated by a rAAV vector is long-termed because of its incorporation into the host genome [196] and currently the vector of choice for the sustained transduction of photoreceptor cells [8].

Lentiviral vectors are capable of stably transducing non-dividing cells [200] and have gained recognition as a relative specific vehicle for the transduction of retinal pigment epithelial cells when subretinal delivered [8,201]. Further advantages of lentiviral vector systems over other vectors consist of the ability of long-lasting transgene expression and the absence of either toxicity and immune or inflammatory response [200]. Because of concerns about dangerous recombination events to generate a fully replication-competent virus, especially in case of human immunodeficiency virus, other lentiviral vectors like feline immunodeficiency virus [202], simian immunodeficiency virus [203,204] and equine infectious anaemia virus [205] are employed which are non-pathogenic to humans [8,200]. Additionally, the "third generation" of lentiviral vectors was developed, which minimises the possibility of recombination through splitting the viral genome in different transcriptional units [200].

Retroviral vectors are capable of transducing dividing cells, but the gene transfer is less efficient than that mediated by adenoviral vectors. They are characterised as safe with no unexpected toxicity, and only for the replication deficient herpes viruses cytoxicity was proven [206,207]. Retroviral vectors insert genes randomly, which may be a problem in specific cases. Retroviral vectors are employed for suicide-gene approaches to proliferative and neoplastic intraocular disorders, but their utility in the eye is limited. However, Kimura and colleagues demonstrated the possibility of gene therapy for PDR in a rabbit model using retroviral vectors [208]. Murata and associates found that the number of proliferating endothelial cells in PDR is relatively small, therefore, they induce proliferation in the retina by photo-coagulation and transducing genes exclusively into photo-coagulation sites. They proposed the combination of photocoagulation and simultaneous transduction of genes encoding anti-angiogenic factors in the therapy of diabetic retinopathy [209].

Each of the aforementioned viral vectors has the ability of delivering cDNA, DNA, antisense mRNA, ribozymes and RNA interference [194]. In the gene therapy of ocular diseases these vectors should deliver either transgenes encoding anti-angiogenic molecules or transgenes that are able to inhibit the expression of angiogenic genes in order to prevent neovascularisation. Since diabetic retinopathy is a chronic disorder, therapeutic effects can be achieved only by long-term expression of the transgene [209]. At last, a successful transfection of specific tissues of the eye is dependent of the optimal choice of vector and the site of its intraocular administration. The expression of the transgene can be targeted now through the use of specific promoters, therefore diminishing side effects through exclusion of effects on neighbouring tissues [194].

To date, several transgenes were introduced into the eye in order to decline neovascularisation or to prevent it. Since it is known that the balance of angiogenic and anti-angiogenic factors, predominantly VEGF and PEDF, play a crucial role in the pathological growth of blood vessels in the eye, these two cytokines were mainly involved in the development of ocular gene therapy.

Bainbridge's group predominantly examined the inhibition of VEGF. They observed a significant reduction of neovascularisation by local gene transfer of the soluble VEGF receptor sFlt-1 in a mouse model [53]. Lai and colleagues confirmed this result in their study with rats [210]. The same research group found a significant reduction of corneal neovascularisation after injection of adenovirus vector expressing antisense VEGF RNA [211]. Recently, Bainbridge and co-workers identified a small VEGF-derived peptide named EG3306 that was as effective as soluble Flt-1 in suppressing ischaemic retinal neovascularisation [212]. In contrast, Mori *et al.* were interested in the angiostatic effect of PEDF. They conducted a study in which an adenovirus delivered PEDF after subretinal or intravitreous injection. In mice with laser-induced rupture of Bruch's membrane, CNV was significantly lower than in control groups. In two other models, in ROP-mice and VEGF overexpressing mice, intravitreous injection of adenovirus-PEDF also resulted in significant reduction of neovascularisation [213,214]. In addition, the use of rAAV vectors to deliver PEDF was as

successful as the use of Ad vectors in inhibiting the growth of pathologic blood vessels in mouse CNV model, and PEDF gene transfer even caused regression of neovascularisation [215]. The same group investigated the periocular injection of adenoviral vector expressing PEDF and found a decrease in CNV [216]. Raisler and colleagues demonstrated beneficial effects of rAAV-vector expressing PEDF on neovascularisation in the neonatal mouse eye, thus confirming the results of Mori and co-workers [217].

Additional angiostatic proteins successfully examined in gene therapy for angiogenic eye diseases are angiostatin [217-219], endostatin [220], tenomodulin [221] and tissue inhibitor of metalloproteinases-3 gene [182,222] as well as tissue inhibitor of metalloproteinase-2 gene [223]. Furthermore, other promising approaches of inhibiting neovascularisation were published recently, among them the development of pseudotyped AAV vectors [224], the subretinal delivery of small interfering RNA directed against VEGF in mice [225], and corneal injection of naked plasmid DNA encoding sFlt-1 [226]. Regulation of transgene expression remains an important feature when using gene therapy in humans. To date, scientists have developed a rapamycin-inducible system [227], the tetracycline-inducible system [228,229] and the incorporation of a hypoxia response element (HRE) into promoter sequences of therapeutic constructs, resulting in transgene expression in response to hypoxia [230].

Table 2. Therapeutic Strategies for the Treatment of Angiogenic Eye Disease

Standard treatment	**Metabolic control, retinal photocoagulation, vitrectomy**
Experimental strategies	
Systemic therapies	
Inhibition of aldose reductase	Sorbinil Tolrestat Epalrestat Fidarestat
Inhibitors of protein glycation	Aminoguanidine pyridoxamine
Antioxidants	vitamin A vitamin C vitamin E
Inhibitors of PKC	LY333531 LY379196 PKC412
Inhibition of GH/IGF-1 axis	Somatostatin analogues: octreotide somatuline GH receptor antagonist: pegvisomant
Local therapies	
Inhibition of VEGF	RhuFab VEGF EYE001
Steroid compounds	Triamcinolone acetonide Fluocinolone acetonide anecortave acetate
Gene therapy	Recombinant virus vectors delivering transgenes encoding angiostatic molecules VEGF antagonists, PEDF
Stem cell therapy	Delivery of endothelial precursors transfected with angiostatic molecules T2-TrpRS

Several strategies of gene therapy succeeded in inhibiting ocular neovascularisation in animal models. Despite these numerous advances, further work has to be performed in the areas of vector toxicity and immune response to achieve a safe alternative in the treatment of eye diseases. First approaches in the prevention of disorders in human eyes are ongoing. This time a phase I study is being performed by Rasmussen and co-workers, in order to assess the safety, tolerability and feasibility of direct intravitreal injection of adenovirus vector encoding PEDF (AdPEDF) in patients with AMD. Furthermore, an appropriate dose range for phase II testing of AdPEDF should be identified [231].

4.2.2.3. Stem Cell Therapy

Recently, a new therapeutic strategy for the treatment of ocular disorders was developed, based on the usage of stem cells. Thereby stem cells with their regenerative potential should promote angiogenesis in the degenerative retina or block it in proliferative eye diseases. It was demonstrated that the bone marrow contains haematopoietic stem cells (HSCs) which can serve as blood vessel precursors during retinal neovascularisation [232]. These stem cells can differentiate along haematopoietic and non-haematopoietic lineages. Otani and colleagues found that non-haematopoietic stem cells contain a population of endothelial precursor cells (EPCs) that can promote angiogenesis by targeting activated astrocytes [233]. These glial elements guide the EPCs into the developing vasculature without disrupting retinal structure. In a model for retinal degeneration, intravitreal injection of HSCs resulted in a nearly normal retinal vasculature in neonatal mice as well as in a rescue of degeneration in adult mice. Ocular administration of HSCs transfected with T2-tryptophanyl-tRNA synthetase, an inhibitor of retinal angiogenesis, was shown to inhibit the formation of normal retinal vasculature in neonatal mouse eyes [233]. It would be interesting to know if this inhibitory effect could be also achieved in adults with abnormal proliferation of retinal vasculature. Additionally, inhibition of neovascularisation under ischaemic conditions may exacerbate the progressing ischaemia [234]. In conclusion, the intraocular administration of stem cells offers an elegant possibility of delivering anti-angiogenic drugs to the places of neovascularisation and gives hope for the development of an effective therapy of PDR.

CONCLUSIONS

To date, retinal photocoagulation treatment and vitrectomy are standard therapies in angiogenic eye disease. Furthermore, a tight metabolic and blood pressure control is mandatory in diabetic patients. Nevertheless, an excellent glycaemic control is difficult to obtain, and many patients become blind despite the availability of effective therapies. Especially in the prevention and early stages of diabetic retinopathy competent strategies are required to overcome this disease as one of the major causes of blindness in the

Western world. Therefore other currently examined approaches such as inhibition of growth factors, specific hyperglycaemia-associated biochemical changes and signalling pathways as well as targeting extracellular matrix and integrin factors might be effective therapeutic options in the course of angiogenic retinopathy (Table 2).

ABBREVIATIONS

ACE = Angiotensin-converting enzyme
Ad = Adenoviral vector
AGE = Advanced glycation end products
AMD = Age-related macular degeneration
ARI = Aldose reductase inhibitor
ATP = Adenosine triphosphate
CML = N-E-carboxymethylated lysine
CNV = Choroidal neovascularisation
DAG = Diacylglycerol
DR = Diabetic retinopathy
EGF = Epidermal growth factor
eNOS = Endothelial nitric oxide synthase
EPC = Endothelial precursor cell
FGF = Fibroblast growth factor
GAP-DH = Glyceraldehyde-3-phosphate dehydrogenase
GH = Growth hormone
HIF-1α = Hypoxia-inducible factor-1α
HRE = Hypoxia response element
IDDM = Insulin-dependent diabetes mellitus
IGF-1 = Insulin-like growth factor 1
IGFBP-1 = Insulin-like growth factor binding protein 1
MAP = Mitogen-activated protein
MAPK = Mitogen-activated protein kinase
MMP = Matrix metalloprotease
NO = Nitric oxide
PAI-1 = Plasminogen activator inhibitor 1
PEDF = Pigment epithelium-derived factor
PDGF = Platelet-derived growth factor
PDR = Proliferative diabetic retinopathy
PDT = Photodynamic therapy
PI3 = Phosphatidylinositol 3-kinase
PKC = Protein kinase C
rAAV = Recombinant adeno-associated virus
RAS = Renin-angiotensin system
ROP = Retinopathy of prematury
ROS = Reactive oxygen species
RPE = Retinal pigment epithelium
SSTR = Somatostatin receptor
TGF-β = Transforming growth factor β
TIMP = Tissue inhibitor of metalloprotease
TNF-α = Tumor necrosis factor α
T1DM = Type 1 diabetes mellitus
VEGF = Vascular endothelial growth factor
VEGFR = Vascular endothelial growth factor receptor

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Inhibitors of Ocular Neovascularization
Promises and Potential Problems

Peter van Wijngaarden, MBBS
Douglas J. Coster, MBBS, FRCS, FRANZCO
Keryn A. Williams, PhD

MOLECULAR MEDICINE OFFERS PROMISE FOR THE prevention of vision loss caused by ocular neovascularization in diabetic retinopathy and exudative age-related macular degeneration (ARMD). During the past decade, significant advances have been made in angiogenesis research, such that the understanding about new vessel formation in disease has increased considerably. This knowledge has led to the development of numerous inhibitors of angiogenesis. Among a host of novel therapeutics for ocular neovascularization, 2 inhibitors of the angiogenic agent vascular endothelial growth factor (VEGF)—pegaptanib sodium and ranibizumab—are poised for imminent clinical application. However, the need for repeated intraocular injection of these agents and the potential for local and systemic adverse effects may pose hurdles for these emerging therapies.

Conventional Treatments Have Limitations

The proliferative retinopathies, principally diabetic retinopathy and exudative ARMD, are leading causes of vision loss worldwide, and their prevalence is projected to increase.[1,2] Central to the pathogenesis of both disorders are increased vascular permeability, leading to retinal edema and subretinal fluid accumulation, and the proliferation of new vessels that are prone to hemorrhage. The established therapy for retinal neovascularization in diabetic retinopathy, laser photocoagulation, may be effective in delaying the progression of the disease but lacks specificity and is associated with retinal destruction, causing impaired visual function.[3] Moreover, retinopathy can progress despite the best available treatment.[4] The management of choroidal neovascularization in ARMD has been boosted by the advent of photodynamic therapy, which involves laser ablation of choroidal new vessels with the aid of a photosensitizer administered systemically via intravenous injection. However, photodynamic therapy is only helpful in a subset of neovascular lesions, and repeated treatments are often required.[5] While photodynamic therapy is often effective in ablating established pathological vessels, it does not prevent new vessel formation. Accordingly, there is a need for treatments that selectively target the molecular mediators of ocular neovascularization.

VEGF—An Appealing Target for Inhibition

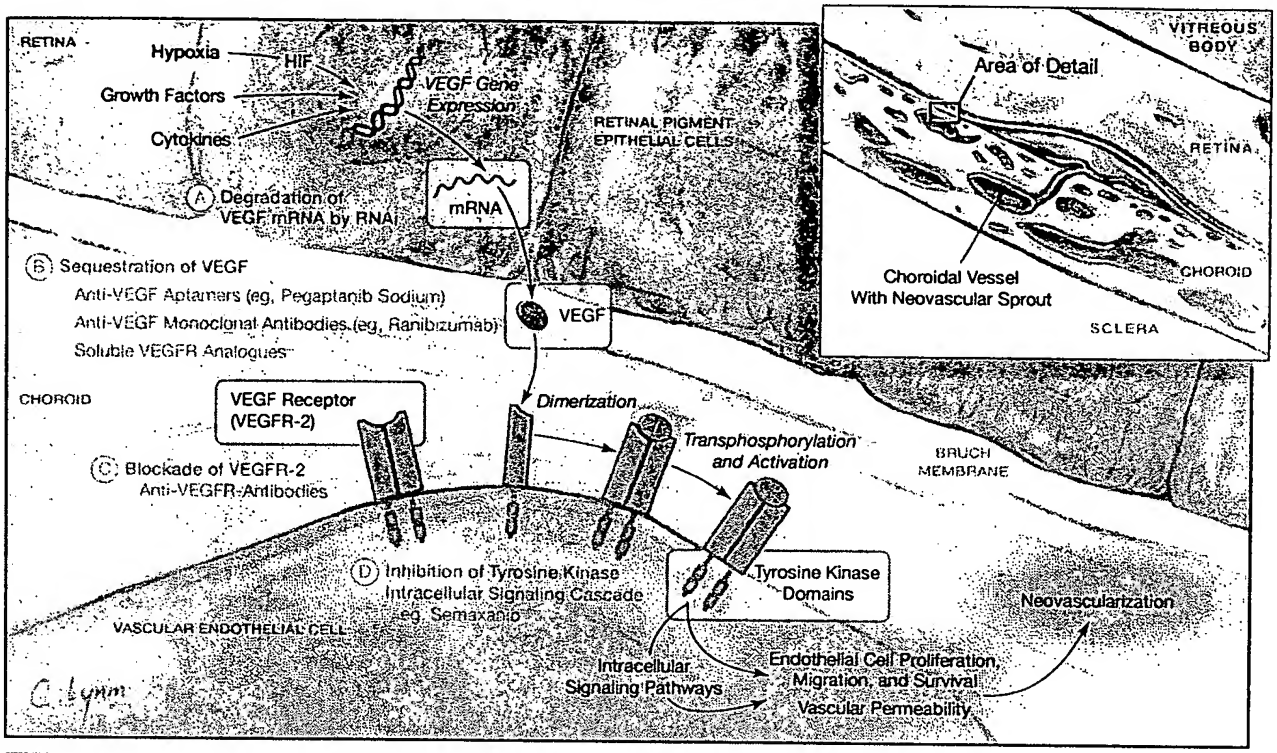
Vascular endothelial growth factor, a central mediator of the complex cascade of angiogenesis and a potent permeability factor, is an attractive target for novel therapeutics. Vascular endothelial growth factor is a peptide growth factor, and alternative messenger RNA splicing gives rise to at least 6 isoforms, of which $VEGF_{165}$ is the major pathogenic species.[6] Vascular endothelial growth factor is the ligand for 2 membrane-bound tyrosine kinase receptors, VEGFR-1 and VEGFR-2. Most of the proangiogenic functions of VEGF are mediated by VEGFR-2. Ligand binding triggers VEGFR-2 dimerization and transphosphorylation with subsequent activation of an intracellular tyrosine kinase domain (FIGURE). The ensuing intracellular signaling axis results in vascular endothelial cell proliferation, migration, and survival.

Vascular endothelial growth factor has been identified in neovascular membranes in both diabetic retinopathy and ARMD, and intraocular levels of the factor correlate with the severity of neovascularization in diabetic retinopathy.[7,8] In the developing human retina, as well as in animal models of proliferative retinopathy, VEGF expression has been temporally, spatially, and quantitatively associated with new vessel formation.[9,10] Therapeutic antagonism of VEGF in these models results in significant inhibition of both retinal and choroidal neovascularization, as well as a reduction in vascular permeability.[11-13]

Several therapeutic strategies are under development to inhibit the activities of VEGF in the proliferative retinopathies (Figure). Approaches involve the sequestration and neutralization of VEGF or the blockade of VEGFR-2. Examples include a VEGF-neutralizing oligonucleotide aptamer (pegaptanib), a humanized anti-VEGF monoclonal antibody fragment (ranibizumab), a receptor analogue (sFlt-1), and a receptor-immunoglobulin fusion protein.[12,14,15] Other strategies are the inhibition of the tyrosine kinase signaling cascade or the degradation of VEGF messenger RNA using small interfering RNAs.[16,17] Of these approaches,

Author Affiliations: Department of Ophthalmology, Flinders University of South Australia, Adelaide, Australia.
Corresponding Author: Peter van Wijngaarden, MBBS, Department of Ophthalmology, Flinders Medical Centre, Bedford Park, Adelaide 5042, Australia (vanw0009@flinders.edu.au).

Figure. Inhibitors of the Vascular Endothelial Growth Factor (VEGF) Signaling Pathway in the Proliferative Retinopathies



Binding of the VEGF to its receptor induces an intracellular signaling cascade that leads to such events as vascular endothelial cell proliferation, migration, and survival, and increased vascular permeability. In the proliferative retinopathies, inhibitors of VEGF may act at various levels of this signaling pathway to exert a therapeutic effect. HIF indicates hypoxia-inducible factor; mRNA, messenger RNA; RNAi, RNA interference.

pegaptanib and ranibizumab have shown promise in clinical trials in patients with exudative ARMD.

Pegaptanib Sodium—An Anti-VEGF Aptamer

Pegaptanib is an RNA oligonucleotide of 28 bases in length with extremely high affinity, in the picomolar range, for the human $VEGF_{165}$ peptide.[18] It binds to $VEGF_{165}$ by a combination of charge and shape complementarity, sequestering it and preventing VEGF receptor activation. The aptamer has demonstrated significant inhibition of vascular permeability and retinal neovascularization in animal models.[19] In a randomized, double-masked, placebo-controlled multicenter phase 3 clinical trial,[18] 1208 patients with exudative ARMD were randomized to receive either intravitreal pegaptanib (0.3 mg, 1.0 mg, or 3.0 mg) or a sham subconjunctival injection every 6 weeks for 48 weeks prior to rerandomization at 54 weeks. At 54 weeks, each patient group demonstrated a progressive loss of vision, but the extent of this loss differed among groups. Treatment with pegaptanib (0.3-mg dose) was associated with a 15% benefit over sham in terms of the primary efficacy end point—the loss of less than 15 letters of visual acuity, as measured with the Early Treatment of Diabetic Retinopathy Study (ETDRS) chart, at 2 m.[18] Paradoxically, the high-dose pegaptanib treatment group (3 mg) was not significantly different from the control group for the efficacy end point. While most trial participants continued to lose vision, fewer patients in the 0.3-mg treatment group experienced severe vision loss (≥30 letters ETDRS) than in the control group (10% vs 22%, $P<.001$).[18] Pegaptanib was approved by the US Food and Drug Administration for the treatment of exudative ARMD in December 2004.[20] At a price of $995 per injection,[21,22] the annual drug cost per patient is approximately $8600, assuming a 6-week dosing schedule.

Ranibizumab—An Antibody Fragment Inhibitor of VEGF

In contrast to pegaptanib, ranibizumab is a recombinant humanized monoclonal antibody fragment with specificity for all isoforms of human VEGF.[23] Ranibizumab demonstrates high affinity for human VEGF and exerts its neutralizing effect by inhibiting the VEGF-receptor interaction. Unlike the larger whole antibody, ranibizumab can penetrate the internal limiting membrane and reach the subretinal space following intravitreal injection in rhesus monkeys.[12,24] In nonhuman primate studies of laser-induced choroidal neovascularization,

intravitreal injection reduced the incidence of new vessel formation as well as leakage from established vessels.[12]

In a phase 2 trial of the antibody fragment in human ARMD, patients were randomized to receive either usual care or intravitreal injections of 300 μg or 500 μg of ranibizumab every 28 days for 4 doses.[25] Ranibizumab treatment was associated with few adverse events aside from reversible ocular inflammation. Visual acuity, as measured with the ETDRS chart, improved 8.5 (3.3) and 12.8 (3.4) (mean [SD]) letters from baseline in the group treated with the 300-μg dose at days 98 and 210, respectively. Patients in the usual care group demonstrated a decrease of 3.0 (5.6) letters at day 98, but improved to a gain of 7.3 (6.6) letters at day 210 following crossover to ranibizumab treatment. Ranibizumab is currently under study in phase 3 clinical trials.

Challenges of Intraocular Drug Delivery

A major limitation of both treatments is the need for repeated intraocular injection. Intravitreal injection is invasive, with the potential for blinding sequelae such as endophthalmitis and retinal detachment. In clinical trials, administration of pegaptanib was associated with an overall risk of endophthalmitis of 0.16% per dose, or 1.3% per patient per year.[18] The incidence of endophthalmitis was significantly reduced following the introduction of more stringent infection control measures at the time of administration. The rates of retinal detachment and traumatic cataract per injection were 0.08% and 0.07%, respectively.[18] While the incidence of serious complications of intraocular injection is low, cumulative risk exposure may be significant for patients requiring serial treatments over many years. Although patients with advanced disease may tolerate repeated injections, given the hope of improved visual function, it is unlikely that individuals with early proliferative diabetic retinopathy or ARMD would be as likely to do so. Accordingly, attempts are being made to formulate alternative delivery vehicles for these drugs.[26]

Potential Hazards of Systemic VEGF Inhibition

In health, the eye is relatively sequestered from the systemic circulation by the tight blood-ocular barrier; however, breakdown of this barrier is common in neovascular eye disease.[27,28] Thus, while intraocular injection of an anti-VEGF therapeutic may provide relative selectivity for VEGF in the eye, systemic exposure is inevitable. This notion is supported by pharmacokinetic findings in trials of pegaptanib and ranibizumab. In rhesus monkeys, peak plasma levels of approximately 0.4 μg/mL were achieved following bilateral intravitreal injections of 0.5 mg of pegaptanib, and mean levels were in excess of 3 ng/mL 28 days later.[29] In humans, mean plasma levels of the aptamer were approximately 80 ng/mL following a single intravitreal injection of 3 mg of pegaptanib (10 times the recommended dose for intraocular injection) and its plasma half-life was 10 (4) (mean [SD]) days.[30] Similarly, in cynomolgus monkeys, peak serum ranibizumab levels were 150 ng/mL following bilateral intravitreal injections of the drug (500 μg) and the serum half-life was 3.5 days.[31] While it is difficult to extrapolate these values directly to humans, it is likely that serum levels of ranibizumab will be lower owing to different kinetics of release from the ocular compartment and the larger volume of distribution. To put these figures into context, plasma VEGF levels in the healthy human adult are typically less than 100 pg/mL, 2 orders of magnitude lower than the observed mean drug levels.[32] While VEGF concentrations at sites of active angiogenesis are substantially higher, the effects of chronic low-level VEGF antagonism are not well characterized.

Although VEGF antagonists appear to be well tolerated in the short term, a growing body of evidence, from animal and in vitro experiments, hints at the potential for serious systemic adverse effects. In addition to playing a role in pathological neovascularization, VEGF is required for normal wound healing, bone growth, cyclic endometrial development, and placental vascularization. Preclinical studies of an antivascular endothelial growth factor antibody (rhuMAbVEGF)—a recombinant humanized whole antibody closely related to ranibizumab—in young adult cynomolgus monkeys demonstrated physeal dysplasia following biweekly intravenous doses of the antibody, as low as 2 mg/kg.[33] At higher doses (10 mg/kg), uterine and ovarian weights were reduced and corpora lutea were absent, indicating impaired reproductive function. Partial restoration of these changes was noted 4 weeks after the cessation of treatment. Vascular endothelial growth factor plays other vital roles, such as the formation of collateral vessels critical to the viability of ischemic limbs and myocardium.[34]

Because individuals with diabetic retinopathy and ARMD may be at increased risk of cardiovascular and peripheral vascular disease, the implications of long-term systemic inhibition of VEGF could be profound.[35-37] These concerns are compounded by the recent discovery of a doubling in the incidence of serious thromboembolic events in patients with colon cancer receiving intravenous anti-VEGF monoclonal antibodies in combination with 5-fluorouracil, relative to those receiving standard chemotherapy.[38] It is estimated that the risk of such events in patients treated with this agent may be as high as 5%. While this population is unique, both in terms of the level of systemic exposure to the anti-VEGF therapeutic and in terms of comorbid illness, these findings warrant concern for patients receiving chronic anti-VEGF therapy for non–life-threatening ocular disease.

The 54-week safety data from the pegaptanib trial are reassuring: cardiovascular events and all-cause mortality were comparable for the pegaptanib-treated and sham-injected groups. Because individuals deemed to be at high risk of cardiac and cerebrovascular events were excluded from trial participation, it remains to be seen whether similar results can be attained in a real-world population. Trials of ranibizumab are still under way. In view of the theoretical potential for adverse cardiovascular events, the US Food and Drug

Administration has endorsed the need for postmarketing surveillance to determine the long-term safety of pegaptanib. While concerns have been voiced about the rigor of postmarketing surveillance, triggered largely by events surrounding the withdrawal of the cyclooxygenase 2 inhibitor rofecoxib,[39] there is a clear need for greater vigilance and timely reporting of adverse safety outcomes of novel therapeutics.

Beyond the Vascular System—A Neural Role for VEGF

It appears that VEGF has important roles in neuronal function.[40] Vascular endothelial growth factor receptors are widely expressed in the brain and spinal cord, and mice deficient in VEGF have a phenotype analogous to the neurodegenerative disorder amyotrophic lateral sclerosis. In addition, the motor neuron degeneration observed in a well-established mouse model of amyotrophic lateral sclerosis (superoxide dismutase with Gly93Ala substitution [$SOD1^{G93A}$] mice) can be significantly delayed by the induction of VEGF expression.[41] Vascular endothelial growth factor has recently been implicated in the proliferation of neuronal stem cells in the murine hippocampus and is thought to play an important role in memory and learning.[42] The factor serves a protective role in acute neuronal ischemia and stimulates the proliferation of a wide range of neuronal and glial cell types in vitro and in vivo.[40] Vascular endothelial growth factor expression has been detected in all classes of neurons and glial cells in the disease-free human retina.[43] It has been postulated that the basal expression of VEGF by the neural retina, which may approach 15 to 20 pg/mg of protein, may serve a role in maintaining retinal vascular homeostasis; however, the potential for an autocrine or paracrine neuroprotective role remains.[44] In vitro experiments suggest that VEGF plays a role in photoreceptor differentiation and may contribute to photoreceptor survival.[43] Granted that photoreceptor degeneration is a key pathological event in ARMD and neuronal ischemia is central to diabetic retinopathy, it remains to be seen whether VEGF antagonists will accelerate these processes in the long-term.

Conclusion

Drugs targeting VEGF offer promise as sight-saving therapies for individuals with advanced diabetic retinopathy and exudative ARMD, for whom other therapeutic interventions are limited. However, enthusiasm for these agents must be tempered by recognition of the potential for significant local and systemic adverse sequelae. While an intraocular excess of VEGF can contribute to disease, the factor also serves numerous essential functions in extraocular tissues. It follows that the ability to selectively localize an anti-VEGF therapeutic to the intraocular environment may be critical to its clinical success. Moreover, as VEGF may serve roles in the maintenance of the neural retina and functional retinal vessels, greater therapeutic precision could be afforded by targeting the downstream effectors of VEGF-signaling that are specific to angiogenesis. Further advances in the understanding of the molecular bases of pathological angiogenesis will lead to the design of therapeutic agents with the potential to ease the burden of neovascular eye disease. Such developments should go hand in hand with therapies targeting the broader spectrum of pathological events in these disorders.

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EDITORIAL

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Physician Substance Abuse and Recovery

What Does It Mean for Physicians—and Everyone Else?

David R. Gastfriend, MD

THE 10% TO 15% PREVALENCE OF SUBSTANCE USE DISorders among physicians is similar to that in the general population,[1,2] but the quality and intensity of treatment given to physicians may far exceed that available to other individuals with these disorders.[3-5] Recognition of the impaired physician began to emerge only in the 1970s[6] and has led to the development of physician health programs (PHPs). These are now mature models, available in many states, usually through medical societies, as an alternative to monitoring by state government boards of registration in medicine.[7] In many cases, physicians who voluntarily contract with a PHP may remain anonymous[7] to the state medical board and the National Practitioner Data Bank, a feature designed to promote early intervention in the disease process, ie, before patients are harmed. Many PHPs now offer services to other health professionals also. Treatment in these programs is probably the most compre-

See also p 1453.

Author Affiliations: Addiction Research Program, Department of Psychiatry, Massachusetts General Hospital, and Department of Psychiatry, Harvard Medical School, Boston, Mass. Dr Gastfriend is now vice president of medical affairs, Alkermes Inc, Cambridge, Mass.
Corresponding Author: David R. Gastfriend, MD, Alkermes Inc, 64 Sidney St, Cambridge, MA 02139 (david.gastfriend@alkermes.com).

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Setting sights on the treatment of ocular angiogenesis using antisense oligonucleotides

Scott P. Henry, Eric G. Marcusson, Thomas M. Vincent and Nicholas M. Dean

Isis Pharmaceuticals, 2292 Faraday Avenue, Carlsbad CA 92008, USA

The application of antisense technology to study physiological and disease processes continues to mature. Antisense approaches are among the most direct means to use genomic sequence information. When developing therapeutics, applications range from early target validation in discovery to the therapeutic product. In this review, we describe the application of antisense oligonucleotides (ASOs) to identify genes that are important in controlling angiogenesis. High-throughput assays *in vitro* have been used to evaluate many gene targets. Genes that appear to be important in angiogenesis are then evaluated further in animal models of ocular angiogenesis. The ability of ASOs to reduce target-gene expression in the appropriate cells in the eye raises the possibility that this class of compounds could be used for target validation *in vivo*, and also be developed as a novel class of therapeutics in their own right.

Target validation (or gene functionalization) is a process used by scientists in an attempt to assign a functional role to genes in selected disease processes. The goal is to exploit the information available from the human genome sequence to understand disease mechanisms and find new drug targets [1]. There are several ways to perform target validation, including comparative gene-expression profiling using DNA and RNA arrays [2,3], the use of knockout models [4], and the pharmacological inhibition of either the activity or the function of specific genes [1,5]. In this review, we focus on the general pros and cons of implementing antisense technology in target validation using the area of angiogenesis as an illustration. We place emphasis on the *in vitro* screening methods that are in use currently and on validation *in vivo*. The focus is on therapeutic applications, but the same principles can be applied to the study of basic physiology and cell biology (see [6–8] for examples in ocular physiology).

Practical considerations in the application of antisense approaches

Among the most efficient tools for target validation are antisense approaches, including RNase H and small-interfering RNA (siRNA) mechanisms, that inhibit the expression of specific proteins [9–11]. As described previously in *TiPS*, targeting mRNA using antisense oligonucleotides (ASOs) is a versatile, efficient way to inhibit gene expression and study cell signaling [12]. An inhibitor can be developed for any gene for which a partial gene sequence is available, and the automation of oligonucleotide synthesis and activity screening in cells in culture enable this process to be completed in a couple of weeks. The types of genes that can be targeted by inhibiting RNA translation are not limited by traditional drug-discovery platforms that, typically, include small molecules designed to inhibit enzyme activity, and protein therapies that, typically, act on either cell-surface or secreted proteins. The primary challenge comes in targeting genes that are expressed only in specialized cells that are difficult to culture and transfect. Targeting RNA also provides specificity and efficiency of drug design that is not available with other validation and discovery platforms, and enables selective inhibition of closely related genes [12]. The ability to rapidly design, synthesize and test specific inhibitors of gene expression provides powerful tools for the target-validation process.

Other target-validation technologies that focus on inhibition of gene expression include the development of mice with single-gene knockouts [13]. Although viable, this approach can be time-consuming and expensive. It is also somewhat limited to the investigation of genes that are not crucial for development. Another limitation of knockout technology is that the gene cannot be manipulated to determine how much inhibition is needed to provide a particular phenotype. However, knockouts do have an advantage over antisense approaches because the gene is absent in all tissues, whereas oligonucleotides are not taken up equally by all tissues.

Some details of targeting RNA that are unique to small-molecule and antibody approaches must be taken into consideration in the successful application of all ASO strategies. First, the design of the optimal ASO inhibitor is an empirical process that requires the evaluation of multiple inhibitors for each gene. Although this sounds daunting, it can be accomplished with relative ease and affordability using modern techniques [14]. Second, although cellular uptake is largely independent of sequences, it does vary between cell types and must be optimized for each set of experimental conditions to ensure that sufficient ASO is available to bind the

Corresponding author: Scott P. Henry (shenry@isisph.com).
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mRNA. Again, this process is reasonably straightforward given the many transfection reagents that are available commercially and the published methods of treatment *in vitro*, but it cannot be taken for granted. The efficiency of cellular uptake can be documented with either labeled oligonucleotides or analytical techniques. The issue of cell uptake must also be considered *in vivo*. Parenteral administration of ASO results in distribution to and uptake by multiple cell types, but some tissues, for example the brain and skeletal muscle, are difficult to target [15]. In some cases these challenges are overcome by local delivery (e.g. ocular and pulmonary) [16,17]. Third, it must be appreciated that ASO strategies, like any pharmacological intervention, can affect cellular physiology by hybridization-independent mechanisms. These effects are likely to result from the ASO binding to cellular proteins by, for example, ionic interactions [18,19]. As with traditional pharmacology, these effects are dealt with by the judicious use of dose–response curves, multiple ASO inhibitors and appropriate control oligonucleotides [14]. Hybridization-independent effects tend to be less potent than the desired effect and, therefore, do not interfere with the interpretation of results from properly designed studies. Using other techniques when possible to confirm the activity of a gene target once it has been identified using ASOs can avoid the potential issues raised by hybridization-independent effects. Thus, ASOs are used widely in the process of target validation.

Once a target for a particular disease is validated, an ASO can also be considered as a therapeutic agent. This is a complex decision that needs to be considered carefully based on many variables, including the quality of the target validation data, relevance of the animal models for human disease and clinical-trial design. Once a gene target has been validated, further characterization is advisable to understand all aspects of the desired activity, potency, tissue distribution, clearance and metabolism, formulations, routes of administration, dose regimens, tolerability, and therapeutic index. These are important whether the desired inhibitor is an ASO or small-molecule inhibitor and, if evaluated thoroughly, can increase the likelihood of clinical success. We discuss ocular angiogenesis as an example of a disease process with many molecular targets that could be regulated by local therapy with ASOs.

Efficient production of potent and selective inhibitors

The production of optimal ASO inhibitors depends on several factors including RNA structure, which is difficult to predict. Typically, this is accomplished through an empirical process in which 40–80 different antisense inhibitors are designed to hybridize with many regions of the target RNA. The most effective antisense inhibitors are selected by transfecting the ASOs into cells and determining which inhibitors best reduce the concentration of the target mRNA [14]. This method identifies highly potent antisense inhibitors of genes of interest in as little as one week. The most effective ASO for each gene is placed in a library of antisense inhibitors, which is used subsequently for functional screening.

Implementing this technology for gene functionalization studies depends on the rapid production of dozens or hundreds of specific inhibitors. Because ASOs are synthesized chemically, they are amenable to automated, high-throughput synthesis and purification techniques. Systems have been developed that enable the synthesis of up to 96 oligonucleotides in parallel, with multiple reagent ports that greatly improve the efficiency of this process [20]. Even commercially available instruments can rapidly synthesize many ASOs. Thus, the synthesis of inhibitors is not a limiting factor in this technology.

High-throughput functional assays

Gene functionalization requires that a system be developed where the effects of inhibitors of many genes can be evaluated rapidly and reliably. The process of target validation is different in every organization and each disease indication. Our approach is to select genes that are likely to have important functions using data from published literature, gene-expression and proteomic data from diseased individuals or disease models, and from in-house, empirical data. Because disease processes are complex and associated with a broad spectrum of changes, ultimately, they produce hundreds of potential candidates for therapeutic intervention. This leaves an investigator wondering which are crucial for disease modulation and which are simply a consequence of other changes. Testable hypotheses can be generated, based on knowledge of implied gene function of the pathways involved, but it is difficult to draw firm conclusions. Typically, the second step is the development of high-throughput functional assays to screen inhibitors efficiently.

With potentially hundreds of genes to test, screening assays must strike a balance between achieving a biologically relevant endpoint and enabling rapid assessment. Using the ASO paradigm, inhibitors of each gene can be synthesized using only sequence information. In one example, investigators have used a reporter gene assay to screen potential targets in chronic neuropathic pain [21]. Other approaches develop several *in vitro* assays with phenotypic properties that are characteristic of a specific indication [22]. Such assay systems have been developed to study, for example, angiogenesis, oncology, metabolic disease and inflammation. In each case, the general approach is to examine several assays that assess functional changes in cells using a 96-well format that enables relatively rapid, high-throughput evaluation. Data are evaluated by correlating the observed phenotypic changes with decreases in target-gene expression relative to treatment with vehicle and control oligonucleotide.

To identify genes that are important in angiogenesis, we developed assays that used primary human umbilical vein endothelial cells (HUVECs). The primary screening assays included measures of cell function and gene expression of four genes that are thought to be important in angiogenesis [β3-integrin, matrix metalloproteinase 4 (MMP14), endoglin and tumor endothelial marker 5] (Figure 1). Positive and negative control ASOs were included in each experiment. Active inhibitors are studied further in secondary assays (Figure 1). Although secondary assays are more difficult than the primary assays, they have the potential to deliver more-valuable data. A

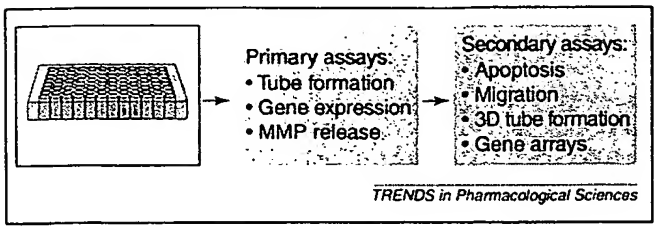

Figure 1. Primary and secondary *in vitro* assays used to screen potential inhibitors of angiogenesis. The primary assay is used to evaluate the activity of antisense oligonucleotide inhibitors of different genes. Those that demonstrate activity progress to the secondary assay. If activity is observed in the secondary assay then an inhibitor might progress to validation in animal models of disease. Abbreviation: MMP, matrix metalloproteinase.

schematic diagram of the process of design, synthesis, lead identification and the flow through the functional assays for an ASO is shown in Figure 2. Inhibitors that block development of the angiogenic phenotype in secondary assays are then validated further in animal models.

The objective is to identify genes that are integral to a disease process and, thus, provide tools to study the disease mechanism and enable therapeutic intervention. However, negative data are also useful because they discount the involvement of many genes in disease processes. In the case of angiogenesis, inhibitors of ~650 genes have been through the primary screening process. Of these ASOs, only a small fraction produced the desired phenotypic changes. Interestingly, enzymes of the Rho-family of GTPases and mitogen-activated protein (MAP) kinase cascade are highly represented. Genes that appear to be important in angiogenesis can be evaluated further in animal models to more thoroughly validate their role in the disease process. Animal studies are more labor and resource intensive, and thus the screening *in vitro* has the potential to save time and expense.

Validation in animal models

Because of the complexities of intact biological systems, it is considered necessary to demonstrate either activity or efficacy in animal models of diseases before a gene is validated fully. In the case of angiogenesis, vascular endothelial growth factor (VEGF), VEGF receptors and some integrins are targets that are already considered validated [23–25]. In particular, VEGF signaling appears to be important in the initiation and progression of angiogenesis, and both antisense and siRNA strategies have been used to confirm this in the eye [26,27]. However, targeting a single growth factor inhibits only one of several potential growth factors that are involved in angiogenesis. Also, it is more focused on the early events of angiogenesis and is not necessarily effective against the terminal phases of angiogenesis. Thus, there is interest in determining whether cell-signaling targets might inhibit many aspects of angiogenesis and complement anti-VEGF strategies. For example, both VEGF and αvβ3-integrin signal through the MAP-kinase pathway [28–30]. Because VEGF provides a signal that initiates angiogenesis and αvβ3-integrin provides the signal necessary for terminal differentiation, inhibiting a signal cascade that is common to both should be beneficial. Small molecules have been used to inhibit signaling [31,32].

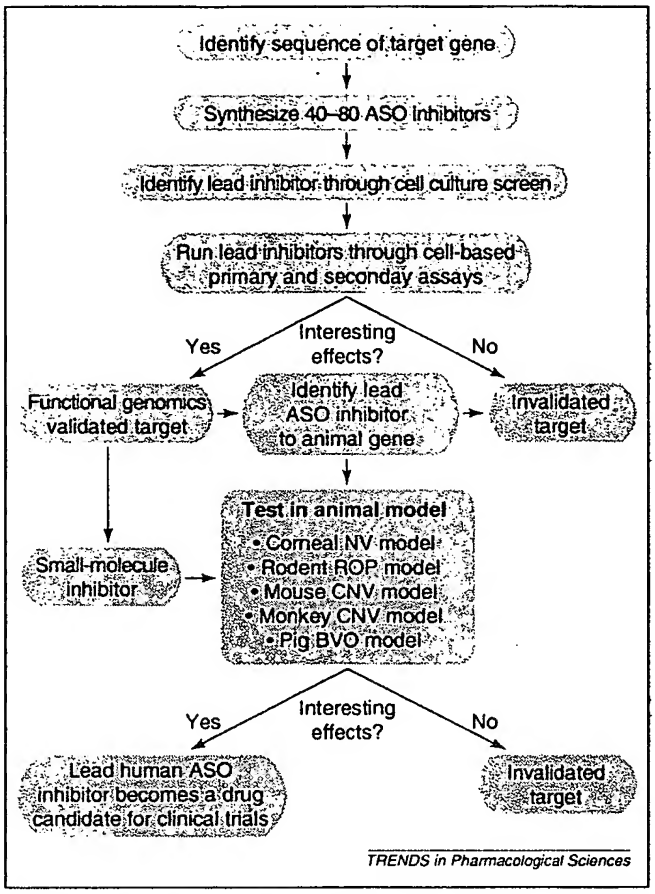

Figure 2. The processes of design, synthesis, lead identification and the flow through the functional assays for an antisense oligonucleotide (ASO) inhibitor. Once an ASO inhibitor is validated by *in vitro* assays, either the ASO inhibitor or an alternative inhibitor (either a small molecule or monoclonal antibody) can be developed and evaluated in an animal model. After validation in an animal model, the inhibitor is considered a drug candidate. Abbreviations: BVO, branched vein occlusion; CNV, choroidal neovascularization; NV, neovascularization; ROP, retinopathy of premat urity.

Inhibiting signaling pathways that are important in different phases of angiogenesis might also be more robust than targeting a single growth factor. At the very least, this approach might provide an effective adjuvant to anti-VEGF therapy. Evaluation of ASOs in animal models has focused on ocular angiogenesis for several reasons. First, retinal and choroidal angiogenesis are more purely related to ocular-disease processes than cancer models, which have many other factors. A second reason is the ability to apply ASOs locally, to the area of angiogenesis. Through immunolocalization and tissue dissection we know that intravitreal injection of oligonucleotides results in efficient uptake into many different cells in the retina and choroid [33]. The third reason is our ultimate interest in applying ASOs therapeutically. The most widely characterized ASO for *in vivo* applications use an RNase H mechanism of action, and are chemically modified to facilitate acceptable tissue distribution, sufficient residence-time in tissues, and reasonable routes of administration [34].

Of the 650 targets identified in the screening assays described above, four genes were selected for further validation in animal models, and these studies are

underway. Following intravitreal injection, ASOs for C-Raf kinase, β3-integrin and other genes selectively reduce levels of target RNA in mouse eyes by 40–60% compared with controls. There are many animal models of ocular angiogenesis, including models of corneal, retinal and choroidal angiogenesis [35]. The inhibitor for C-Raf kinase has made the most progress in animal models. Other work *in vitro* with the C-Raf kinase ASO illustrates the potential of blocking growth-factor and cytokine signaling in HUVECs [36]. One animal model available for target validation is a mouse model of choroidal neovascularization [37]. The inhibition of C-Raf kinase by the ASO in mouse eyes correlates with a modest, statistically significant, decrease in the area of neovascularization (S.P. Henry *et al.*, unpublished). An inhibitor of C-Raf kinase has also been examined in a pig model of branched vein occlusion retinal neovascularization [32]. Similar to the results in the mouse model, this ASO decreased the concentration of C-Raf kinase RNA and protein, and decreased retinal neovascularization [38]. Thus, C-Raf kinase has been validated in animal models and has demonstrated potential as an antiangiogenic therapy [32]. Other inhibitors of the MAP-kinase-signaling pathway are being evaluated.

Therapeutic application of ASO inhibitors

In addition to value as research tools, we are also developing ASO inhibitors as therapeutic agents for ocular disease. An ASO inhibitor of human cytomegalovirus (CMV) has been approved for the treatment of CMV retinitis. This is an important contribution to available therapies because this novel mechanism of action circumvents issues with resistance of the virus to standard therapy [39]. Following approval of the first ASO therapeutic, there has been substantial improvement in the properties of ASO drugs. The primary improvement has come from the use 2′-*O*-methoxyethyl (2′-MOE)-modified oligonucleotides. This modification increases hybridization affinity, which increases potency and slows the rate of metabolic degradation [40,41]. The effect on metabolism greatly extends the half-life of oligonucleotide in cells, where pharmacological activity occurs. Following intravitreal injection, the half-lives in the retina and choroid of rabbits and monkeys is 6–8 weeks. This should enable infrequent treatment [33,42], which is desirable for a local, ocular therapy. The half-life, coupled with the broad cellular distribution in the retina and choroid, is compatible with interest in using ASOs to treat many different ocular diseases, including glaucoma and inflammation [16]. Long half-lives should enable the use of dose regimens (once every other month or longer) without the need for complex formulation or implant devices.

2′-MOE ASOs are also well tolerated in the eye. The use of 2′-alkyl substituents has also contributed to increase tolerability by decreasing the pro-inflammatory effects of ASOs [43]. The substitution of 5-methyl cytosine for cytosine, and avoidance of sequences motifs that are optimal for pro-inflammatory effects has also greatly improved the overall tolerability. Ocular-tolerability studies in rabbits and monkeys in which different ASOs have been administered repeatedly by intravitreal injection for up to 6 months reveal no significant evidence of ocular inflammation.

Preliminary work also indicates that peri-orbital administration can achieve the necessary retinal concentrations for inhibition of target gene expression. In rabbits, retinal concentrations sufficient to reduce target-gene expression were achieved seven days after subtenon injection of ASO in a simple saline formulation (S.P. Henry *et al.*, unpublished). Trans-scleral absorption of other molecules, including ASOs using iontophoresis, and monoclonal antibodies using continuous subconjunctival infusion confirm that the sclera is permeable to medium–large molecules that are highly water soluble [44,45]. Thus, it should be possible to develop a convenient, reliable, non-intravitreal route of administration that would greatly increase the therapeutic utility of ASOs.

Concluding remarks

The successful application of ASOs both *in vitro* and *in vivo*, makes the potential utility of targeting protein expression through RNA clearer. The application of 2′-MOE ASOs is particularly desirable because the same technology can be employed from early screening and selection of gene targets, through to validation in animal models and, finally, to therapeutic investigation in patients. The applications for diseases such as ocular angiogenesis are well suited to this progression because administration is localized and the compounds have desirable pharmacokinetic and safety properties in the eye.

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